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David L. Parker

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Runge et al.

Serial No.: 09/832,069

Filed: April 10, 2001

For: Mitochondrial DNA Damage as a Predictor of Coronary Atherosclerotic Heart Disease

Group Art Unit: 1634

Examiner: Goldberg, Jeanine Anne

Atty. Dkt. No.: CLFR:183US

APPEAL BRIEF

MS Appeal Briefs
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

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Sir:

Appellants hereby submit this Appeal Brief to the Board of Patent Appeals and Interferences pursuant to 37 C.F.R. §41.31(a)(1) and 41.37 in light of the Office Action dated April 5, 2006. It is believed that the filing of the present Appeal Brief is timely, and the appropriate fees for filing this Brief are enclosed. However, if any fees are due for any reason relating to the enclosed materials, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/CLFR:183US.

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I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Research Development Foundation.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 6, 8, 9 and 14-23 are pending and stand rejected. All claims are subject to the present appeal. A copy of the pending and appealed claims is attached as the Claims Appendix.

IV. STATUS OF AMENDMENTS

All previously sought amendments have been entered. Appellants are submitting herewith an amendment to address the minor "antecedent basis" rejection of claim 23 under 35 U.S.C.§112, second paragraph.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The principle claim, claim 6, is directed to a method of measuring the amount of oxidative stress in a human individual, comprising the steps of collecting a blood sample from said individual and assessing the amount of mitochondrial DNA damage in cells from said sample wherein such amount of damage is indicative of oxidative stress in said individual. Specification, page 8, line 19, to page 9, line 16; page 10, lines 8-18; Figure 12; page 18, lines 1-11; Example 19.

Dependent claim 8 is directed to assessing said mitochondrial DNA damage by quantitative PCR. Specification, page 23, lines 2-5.

Dependent claim 9 is directed to assessing the amount of oxidative stress, wherein increased amounts of oxidative stress are predictive of atherogenesis, hypertension, diabetes

mellitis, hypercholesterolemia, degenerative diseases of aging or cancer. Specification, page 24, lines 6-9.

Dependent claim 14 is directed to assessing mitochondrial DNA damage by measuring the amount of DNA damage per length of mitochondrial DNA. Specification, page 23, line 11 – 21.

Dependent claims 15 directed assessing mitochondrial DNA damage that comprises one or more deletions, insertions or duplications. Specification, page 25, line 18, to page 26, line 6.

Dependent claims 16 is directed to assessing mitochondrial DNA damage by measuring mitochondrial mRNA production. Specification, pages 35-37 (Example 5).

Dependent claim 17 is directed to assessing mitochondrial DNA damage by measuring mitochondrial protein production. Specification, pages 37-38 (Example 6).

Dependent claim 18 is directed to assessing mitochondrial DNA damage by measuring changes in mitochondrial oxidative phosphorylation. Specification, pages 38-39 (Example 7).

Dependent claim 19 is directed to assessing mitochondrial DNA damage by measuring changes in mitochondrial ATP production. Specification, pages 38-39 (Example 7).

Dependent claim 20 is directed to assessing mitochondrial DNA damage by measuring changes in mitochondrial redox state. Specification, pages 40-48 (Example 9).

Dependent claim 21 is directed to determining the amount of DNA damage in a nuclear gene in said tissue of interest; and comparing the amount of DNA damage per length of DNA between said mitochondrial DNA and said nuclear gene, wherein a greater amount of

mitochondrial DNA damage per length of DNA than nuclear DNA damage per length of DNA is indicative of an increased amount of oxidative stress in said individual. Specification, page 9, line 17, to page 10, line 7.

Dependent claim 22 is directed to treatment of said DNA with FAPY glycosylase prior to said PCR amplification for detection of 8-oxo-G-lesion. Specification, page 26, lines 1-6; Example 3.

Dependent claim 23 is directed to testing samples of white cells. Specification, page 10, lines 8-18.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There are two rejections that are the subject of the present appeal:

1) A rejection of claims 6 and 16-20 as lacking enablement under 35 U.S.C. §112, first paragraph; and

2) A rejection of claims 6, 8-9 and 14-23 as indefinite under 35 U.S.C. §112, second paragraph

(Appellants presume that the rejection of claim 23 as indefinite on an antecedent basis issue has been adequately addressed in Appellants concurrently submitted amendment.)

VII. ARGUMENT

A. Section 112, First Paragraph Rejections

The Final Action first rejects claim 6 and 16-20 under 35 USC 112, first paragraph, with the Action taking the position that while the specification is enabling for a method for measuring

the amount of oxidative stress by detecting the amount of DNA damage, it does not reasonably provide enablement for detecting mtDNA damage by measuring mt mRNA production, mt protein production, mt oxidative phosphorylation, mt ATP production or changes in oxidative redox state.

In response, Appellants submit that the Action again fails to set forth any cognizable evidence to support its position of non-enablement and, as such, has failed to set forth a *prima facie* case based on substantial evidence as the law requires. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). To make out a *prima facie* rejection, an examiner is required to come forward with evidence or sufficient reasoning substantiating the doubts advanced. *In re Dinh-Nguyen*, 181 U.S.P.Q. 46 (C.C.P.A. 1974). The Examiner attempted to address this issue merely by reference to the Corral-Debrinski *et al.*, 1992, article (Exhibit 1 of Evidence Appendix) as well as what appear to be facts within the Examiner's own personal knowledge, which facts have not been substantiated on this record as required by 37 C.F.R. 1.104(d)(2). The Examiner was invited to submit an affidavit setting forth this knowledge with particularity, but no such affidavit was forthcoming. Thus, it is submitted that any "facts" alleged or relied upon by the Examiner based on the Examiner's personal knowledge must be disregarded. 37 C.F.R. 1.104(d)(2).

Before turning to the specifics of the rejection, Appellants first point out that the real "enablement" question here is whether one of skill in the art can carry out the assay as claimed – *i.e.*, whether one of skill in the could undertake a measurement of mt mRNA production, for example. Yet, there has been no suggestion in the Action that one of skill in the art would not be enabled to carry out such an assay, merely whether such an assay would be reasonably predictive

of mtDNA damage. Thus, the question is perhaps more appropriately considered one of operability/utility.

The Action Fails to Set Forth Evidence of Unpredictability

Turning to the Action's comments, in an attempt to satisfy the *Wands* criteria, the Action first takes the position that that the relationship between mitochondrial ("mt") DNA damage is not quantitatively related to mt mRNA production, mt protein production, mt oxidative phosphorylation, mt ATP production or changes in oxidative redox state, and yet provides no support for this conclusion. Rather, the Action simply states that the "art teaches that tissue ischemia, OXPHOS gene defects, environmental toxins, mtDNA mutations, decreased cellular ATP and oxygen radical formation all affect phosphorylation dysfunction which leads to tissue degeneration and cell death" referring to the article of Corral-Debrinski *et al.*, 1992. Action at page 3-4. The Action then states that "[t]he art does not teach how the amount of mtDNA damage is affected or associated by each of these factors."

Applicant again observes that the statement lifted by the Examiner from the Corral-Debrinki *et al.* article in no way supports a conclusion of non-enablement. Rather, it serves the opposite purpose and indeed supports enablement: Corral-Debrinki *et al.* itself states "[t]his cumulative mtDNA damage was associated with a compensatory 3.5-fold induction of nuclear OXPHOS gene mRNA" and goes on to report a correlation between oxidative stress and elevated mitochondrial damage.[1] So, the Examiner has failed to set forth any cognizable teaching to support her position of non-enablement. Thus, the only "evidence" of non-enablement appears

[1] Of course, a principal advance of the presently claimed invention over Corral-Debrinski *et al.* is that the present claims are directed to measuring such damage in tissues other than heart tissues, which would be an impractical screen for the population as a whole.

to be facts within the Examiner's own personal knowledge, which facts have not been substantiated on this record as required by 37 C.F.R. 1.104(d)(2).

Further, in Appellant's previous response various pieces of scientific literature were presented to rebut the Examiner's statement that the art fails to show a correlation between DNA damage and the aforementioned factors. First, Appellants direct the Board's attention to the Lenaz article (*Biochimica et Biophysica Acta* 1366, 1998, pp 53-67; Exhibit 2 of Evidence Appendix). In its abstract, Lenaz references the "vicious cycle" established between mtDNA damage and altered oxidative phosphorylation and overproduction of reactive oxygen species. The article of Hudson *et al.* (*Free Rad. Res.*, vol 29, pp. 573-579, 1998; Exhibit 3 of Evidence Appendix) references the contribution of mtDNA damage to a decrease in mitochondrial cytochrome c oxidase (COX) activity, associated with a reduction in COX gene and protein expression and a similar decrease in the rate of mitochondrial protein synthesis. See first paragraph, page 573. Lastly, attention is directed to the article of Williams *et al.* (Exhibit 4 of Evidence Appendix), which demonstrates that "altered mitochondrial function" is correlated with increased oxidative damage (by virtue of incorporating 8-hydroxydeoxygunosine into DNA), including "oxygen consumption" and "ATP production." See, *e.g.*, title, first full paragraph, page 28510 and last paragraph, page 28515.

Guidance in the Specification – Working Examples

The Action next concludes that there is insufficient exemplary support to demonstrate that the claimed invention is operable with respect to the various alternative "indirect" methods for measuring mt DNA damage. We submit that the presence or absence of working examples is ordinarily an insufficient basis for finding non-enablement. *Ex parte Nardi*, 229 USPQ 79

(BPAI 1986). This is particularly true where, as here, the question is not whether one of skill can appropriately carry out the assay, it is really whether the assay is reasonably predictive of mtDNA damage.

Nevertheless, we disagree that with the Examiner's position that there is no exemplary support in the specification. We would, for example, direct the Board's attention to Example 5 of the specification, pages 35-37 and Figure 3, which demonstrates the use of Northern transcript analysis to quantify mitochondrial mtRNA transcript levels (claim 16). These studies are discussed in Example 9, at page 44, lines 5-18, and demonstrate a correlation between ND2 and cyt b transcript levels with peroxynitrite treatment. Similarly, with respect to protein production as a measurement of mt DNA damage (claim 17), the Board is directed to Example 6, pages 37-38, which demonstrates an assessment of mitochondrial protein synthesis as a measure of oxidative damage, and provides a reference for exemplary analyses. Similarly, Example 7, pages 38-39, demonstrates assessing oxidative damage by measuring changes is mitochondrial oxidative phosphorylation (claim 18) or mitochondrial ATP production (claim 19), and Example 9, pages 40-48, demonstrate assessing oxidative damage as a function of mitochondrial redox state (claim 20). The significance of the foregoing studies as a measurement of oxidative damage are explained and discussed in Example 9.

In an attempt to support a conclusion of non-operability, the Action states at page 4, that an mtDNA mutation could lead to a protein production of zero and that this would, according to the Examiner, not provide any guidance as to the quantity of DNA damage.

We would respond to this by observing that the examiner's scientific reasoning, which is unsupported by any affidavit or art, is totally unfounded and contrary to what a scientist would

expect, which is that the ultimate expression of a particular mt gene would very definitely be directly related to the amount of random damage in that particular gene. For example, let's say we have a population of 100 mitochondria, each having one gene coding for "X". If 20 of the 100 mitochondria have mutational "hits" in them, one would very definitely expect there to be about a 20% reduced activity of the gene product as compared to a 100 mitochondria control with no such mutational hits. The Examiner's scientific reasoning is very definitely faulty in that she tries to look at a single gene of a single mitochondria rather than a population of mitochondria, which is what is going to be measured in a blood sample!

Next, the Examiner argues, again without any scientific support or affidavit, that some lesions or mutations may occur in non-coding regions and thus would not affect protein production. This analysis, while perhaps applicable to a situation where one is looking at a single mitochondria, again misses the point for essentially the same reason. Where there is a *population* of mitochodria being tested (which there certainly would be if one were testing a blood sample or blood products as stated by the claim), there would necessarily be a vast range of mutations occurring in the mitochondria, some within coding regions and some outside of a particular coding region. Nevertheless, the distribution would be expected to be random and thus demonstrate a readily identifiable *correlation* between the amount of damage in any one gene, as measured by expression of that gene, and the amount of damage overall. The Examiner in fact confirms this by correctly stating that the "knockout of mitochondrial enzyme with a single mutation could cause dysfunction." That's precisely the point: in any population of mitochondria there will be a distribution of gene knockouts by mutation and the level of that distribution of knockouts, as reflected by the activity or amount of the particular mt protein or mRNA being measured will reflect the degree of DNA damage. If *every single* mitochondria in

the sample has that particular mt protein or mRNA knocked out – whether that protein or mRNA is involved in ATP production or mitochondrial redox state – you darn well know that *that* patient has some real problems with their mitochondria!

Lastly, responding to the Examiner's allegation that the specification does not teach a direct tie between mt gene mutations and the activity of mt gene products, we would state that a direct tie between the amount of gene mutational damage and the expression of *any* given gene, mRNA, *etc.*, is, simply put, self evident, and the Examiner has presented no evidence to the contrary.

Quantity of Experimentation

Turning to the next section of the non-enablement rejection, that dealing with quantity of experimentation, the Examiner again fails to come forth with any cognizable evidence and merely states, incorrectly, in a conclusory fashion that there "are many other factors which would affect each of these quantities which may not be related to the amount of mtDNA damage." However, the Examiner notably fails to point to any such "many other factor" in either any scientific literature or in an affidavit as required by 37 CFR §1.104(d)(2).

Thus, as explained above, the Action fails to set forth a *prima facie* case of inoperability of the rejected claims.

B. Section 112, Second Paragraph Rejections

The Action next rejects claims 6, 8-9 and 14-23, stating that the claims are indefinite in that it is said to be unclear whether the final clause of the method is directed to detecting amount of DNA damage or mere presence of damage.

In attempt to address this question and moot the appeal of this rejection, the Examiner was contacted by telephone and asked what amendment the Examiner would like to see to clear up any perceived confusion as to the meaning of the claim. However, the Examiner stated that the section 112, second paragraph, rejection was intimately tied to the section 112, first paragraph rejection, and thus was not amenable to addressing it separate from the section 112, first paragraph, rejection.

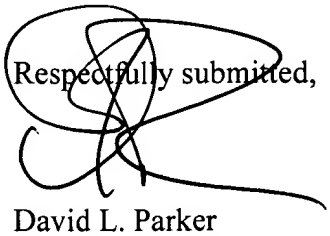
Turning to the subject rejection, Appellants submit that the wording of claim 6 is clear – it concerns "assessing the ***amount*** of mitochondrial DNA damage", wherein the "amount" of damage is indicative of oxidative stress. Thus, it is evident that the claims concern assessing an amount of damage as evidenced by analyzing the DNA directly or by assessing secondary indications of such damage, such as mt RNA production, protein synthesis, *etc.*

Appellants submit that the claims are, on their face, and are in no way indefinite. Accordingly, the Board is requested to reverse the rejection.

CONCLUSION

Appellants believe that the foregoing remarks fully respond to all outstanding matters for this application. Appellants respectfully request that the Board reverse the rejections of all claims.

Respectfully submitted,



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VIII. CLAIMS APPENDIX

1.-5. (Canceled)

6. (Previously presented) A method of measuring the amount of oxidative stress in a human individual, comprising the steps of:

(a) collecting a blood sample from said individual;

(b) assessing the amount of mitochondrial DNA damage in cells from said sample wherein such amount of damage is indicative of oxidative stress in said individual.

7. (Canceled)

8. (Previously presented) The method of claim 14, wherein said mitochondrial DNA damage is assessed by quantitative PCR.

9. (Previously presented) The method of claim 6, wherein increased amounts of oxidative stress are predictive of atherogenesis, hypertension, diabetes mellitis, hypercholesterolemia, degenerative diseases of aging or cancer.

10.-13. (Canceled)

14. (Previously presented) The method of claim 6, wherein said mitochondrial DNA damage is assessed by measuring the amount of DNA damage per length of mitochondrial DNA.

15. (Previously presented) The method of claim 14, wherein the DNA damage comprises one or more deletions, insertions or duplications.

16. (Previously presented) The method of claim 6, wherein said mitochondrial DNA damage is assessed by measuring mitochondrial mRNA production.

17. (Previously presented) The method of claim 6, wherein said mitochondrial DNA damage is assessed by measuring mitochondrial protein production.

18. (Previously presented) The method of claim 6, wherein said mitochondrial DNA damage is assessed by measuring changes in mitochondrial oxidative phosphorylation.

19. (Previously presented) The method of claim 6, wherein said mitochondrial DNA damage is assessed by measuring changes in mitochondrial ATP production.

20. (Previously presented) The method of claim 6, wherein said mitochondrial DNA damage is assessed by measuring changes in mitochondrial redox state.

21. (Previously presented) The method of claim 14, further comprising determining the amount of DNA damage in a nuclear gene in said tissue of interest; and comparing the amount of DNA damage per length of DNA between said mitochondrial DNA and said nuclear gene, wherein a greater amount of mitochondrial DNA damage per length of DNA than nuclear DNA damage per length of DNA is indicative of an increased amount of oxidative stress in said individual.

22. (Previously presented) The method of claim 8, wherein said DNA is treated with FAPY glycosylase prior to said PCR amplification for detection of 8-oxo-G-lesion.

23. (Previously presented) The method of claim 6, wherein cells of the sample are further defined as white cells.

IX. EVIDENCE APPENDIX

Exhibit 1 -- Corral-Debrinski *et al.* article; made of record by the Examiner in the Office Action dated February 15, 2005, at page 4;

Exhibit 2 – Lenaz article; made of record by Appellants in their Amendment and Response dated January 20, 2006 at page 4;

Exhibit 3 -- Hudson *et al.* article; made or record by Appellants in their Amendment and Response dated January 20, 2006 at page 4; and

Exhibit 4 -- Williams *et al.* article; made of record by Appellants in their Amendment and Response dated January 20, 2006 at page 5 (also submitted as reference C48; PTO 1449; July 1, 2005)

EXHIBIT 1

Mutation Research, 275 (1992) 169–180

MUTAGI 0251

Association of mitochondrial DNA damage with aging and coronary atherosclerotic heart disease

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Summary

The role of somatic mitochondrial DNA (mtDNA) damage in human aging and progressive diseases of oxidative phosphorylation (OXPHOS) was examined by quantitating the accumulation of mtDNA deletions in normal hearts and hearts with coronary atherosclerotic disease. In normal hearts, mtDNA deletions appeared after 40 and subsequently accumulated with age. The common 4977 nucleotide pair (np) deletion ($mtDNA^{4977}$) reached a maximum of 0.007%, with the $mtDNA^{7436}$ and $mtDNA^{10.422}$ deletions appearing at the same time. In hearts deprived of mitochondrial substrates due to coronary artery disease, the level of the $mtDNA^{4977}$ deletion was elevated 7–220-fold over age-matched controls, with the $mtDNA^{7436}$ and $mtDNA^{10.422}$ deletions increasing in parallel. This cumulative mtDNA damage was associated with a compensatory 3.5-fold induction of nuclear OXPHOS gene mRNA and regions of ischemic hearts subjected to the greatest work load (left ventricle) showed the greatest accumulation of mtDNA damage and OXPHOS gene induction. These observations support the hypothesis that mtDNA damage does accumulate with age and indicates that respiratory stress greatly elevates mitochondrial damage.

Recently a number of age-related neuromuscular degenerative diseases have been associated with mutations in the mtDNA. Moreover, oxidative phosphorylation (OXPHOS) has been observed to decline with age and has been associated with the accumulation of mtDNA mutations in somatic tissues. These observations suggest that somatic mtDNA mutations may be an important component of aging and the progression of neuromuscular diseases.

The mitochondria generate most of the cellular ATP by the process of OXPHOS. OXPHOS includes five enzyme complexes (I–V). Complexes I–IV encompass the electron transport chain which oxidizes NADH with oxygen and uses the energy to pump protons across the mitochondrial inner membrane. Complex V (ATP

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synthase) exploits this electrochemical gradient as a source of energy to produce ATP. Each of these complexes is composed of multiple polypeptides. 13 encoded by the mtDNA and approximately 60 polypeptides encoded by the nuclear DNA (nDNA).

Hereditary diseases of OXPHOS can result from mutations in either the nDNA or mtDNA OXPHOS genes. The mtDNA has its own independent replication, transcription and translation systems, and the genetic complexity of OXPHOS diseases can be understood through the unique features of mtDNA genetics. Each cell contains hundreds of mitochondria and thousands of mtDNAs (Johnson et al., 1980; Shmookler Reis et al., 1983). The mtDNA is maternally inherited (Case and Wallace, 1981; Giles et al., 1980) and mutations accumulate 10–20 times faster in the mtDNA than in comparable nuclear genes (Neckelman et al., 1989; Wallace et al., 1987). When a cell acquires a mtDNA mutation, this creates a mixed intracellular population of mutant and wild-type molecules known as heteroplasmy. As a heteroplasmic cell undergoes mitotic or meiotic replication, segregation of mtDNA occurs giving rise to homoplasmic cells (pure mutant or wild-type). Different organs and tissues rely on mitochondrial ATP production to different extents. Thus, the number of organs affected and the extent of their clinical manifestations can vary in a family based on the quantity of mutant mtDNA inherited (Shoffner and Wallace, 1991).

A broad spectrum of hereditary neurological and neuromuscular diseases have been ascribed to OXPHOS gene mutations. Nuclear mutations have been demonstrated to cause specific cases of infantile mitochondrial myopathy and Leigh's encephalomyopathy (Zheng et al., 1989; Miranda et al., 1989). mtDNA point mutations have been shown to cause Leber's hereditary optic neuropathy (LHON) (Wallace et al., 1988; Brown et al., 1992), myoclonic epilepsy and ragged-red muscle fibers (MERRF) (Shoffner et al., 1990), neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) (Holt et al., 1990), and mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) (Goto et al., 1990). Mitochondrial deletions and duplications have been shown to cause the chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre (KS) and Pearson's syndromes (Holt et al., 1990; Rotig et al., 1989; Shoffner et al., 1989; Poulton et al., 1989). mtDNA diseases encompass a wide spectrum of clinical manifestations including progressive dementia, myoclonus, and movement disorders. However, virtually all share the common feature that the patients are born normal and acquire symptoms as they age. Hence, mtDNA diseases, like aging itself, have the unique characteristic of a late onset and progressive course.

It has been proposed that the major cause of aging is the accumulation of mtDNA mutations in somatic cells (Linnane et al., 1989). Human oxidative work capacity, as monitored by the anaerobic threshold in exercise stress tests, declines about 1% per year (Astrand et al., 1973). Respiratory complexes I, III and IV all decline with age in skeletal muscle (Trounce et al., 1989) and in liver (Yen et al., 1989). This age-related decline in OXPHOS has been associated with a progressive accumulation of deleted mtDNAs (Cortopassi and Arnheim, 1990; Ozawa et al., 1990; Hattori et al., 1991; Corral-Debrinski et al., 1991). This suggests that the accumulation of somatic mutations in the mtDNA may be a key factor in aging and the progression of mitochondrial diseases.

One agent which is thought to cause the age-related accumulation of mtDNA damage is oxygen free radicals. The respiratory chain is the source of a steady flux of oxygen radicals, including superoxide (O_2^-) (Chance et al., 1979). Superoxide is removed from the mitochondria by the manganese superoxide dismutase, but this reaction produces H_2O_2 which accumulates in the mitochondria. The H_2O_2 can react with superoxide to generate hydroxyl radicals ($OH^\cdot$) which are extremely reactive. Free radicals cause lipid peroxidation, and protein and nucleic acid oxidation resulting in widespread cellular injury (Lippman, 1983; Gutteridge et al., 1985; Miquel et al., 1989). The close proximity of the mtDNA to these reactive molecules in the inner mitochondrial membrane and the deficiency in mtDNA repair systems (Fukanaga and Yielding, 1979) result in preferential oxidative damage to the mtDNA. For example, in rat liver the mtDNA accumulates

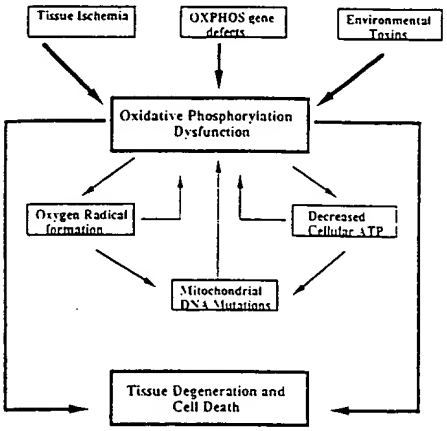

Fig. 1. Proposed OXPHOS model of aging and progression of degenerative diseases. OXPHOS defects initiate a self-perpetuating downward spiral involving electron transport inhibition, reduced energy output and increased oxygen radical production, further damage and greater OXPHOS inhibition.

16-fold higher levels of the DNA oxidation product 8-hydroxydeoxyguanosine than does the nDNA (Ritcher et al., 1988).

These observations suggest a general hypothesis for aging and the progression of degenerative diseases. Oxidative damage to OXPHOS and mtDNA accumulates with age. This reduces mitochondrial energetic capacity, further stimulating oxygen radical production. The resulting mtDNA damage inhibits mitochondrial biogenesis and increases replication errors and mtDNA deletions. Ultimately, sufficient mitochondrial enzyme and DNA damage accumulates, such that the ATP-generating capacity of the cells falls below the minimum energetic thresholds for the cells to function, resulting in failure. The time at which an individual's organs cross these expression thresholds depends on three factors: (1) the level of OXPHOS capacity at birth as determined by the individual's genotype, (2) environmental insults which can inhibit OXPHOS and (3) the accumulation of mitochondrial and mtDNA damage (Fig. 1).

Coronary atherosclerotic heart disease (CAHD) provides an ideal system for studying the role of oxygen radicals on aging and ischemia. One of the main causes of cardiac ischemia is coronary artery stenosis due to atherosclerosis. The resulting episodic ischemia and reperfusion is associated with the generation of free radicals which damage the myocardial tissue. During ischemia the electron transport chain is inhibited, diminishing adenine nucleotide pools and increasing the electronegativity of the electron carriers. This favors the transfer of electrons to molecular oxygen. Concurrently, oxygen detoxification systems decline. During ischemia in the rabbit, there is a decrease in reduced glutathione and a 50% loss of heart mitochondrial superoxide dismutase (Ferrari et al., 1985). Similar observations have been reported in human skeletal muscle during shock (Corbucci et al., 1985). Consequently, ischemia/reperfusion leads to an increase in radical production and a decrease in radical-scavenging capability, resulting in increased mitochondrial, mtDNA and cellular damage and generalized tissue injury and necrosis (McCord, 1988).

We have examined this hypothesis by analyzing the levels of the common 4977 nucleotide pair (np) mtDNA deletion ($mtDNA^{4977}$), and the transcript levels of nDNA and mtDNA OXPHOS genes in normal and coronary atherosclerotic hearts of various ages. This revealed that mtDNA damage accumulates with age in normal hearts, but is dramatically increased in CAHD. Moreover, the increased cardiac mtDNA damage is associated with a compensatory induction of OXPHOS gene expression, particularly the heart-skeletal isoform of the adenine nucleotide translocator (ANT1) (Corral-Debrinski et al., 1991), the protein which exchanges ADP/ATP across the mitochondrial inner membrane (Li et al., 1989). We now extend these observations by demonstrating that coronary atherosclerotic hearts as well as aging hearts accumulate multiple kinds of mtDNA deletions suggesting that a high proportion of mtDNA could be defective in these cells. Further, we report that mtDNA damage accumulates preferentially in the more oxidative areas of the hearts. These results provide additional support for the hypothesis that in the heart, aging and degenerative diseases are associated with increased mtDNA and mitochondrial damage.

Methods

Tissue procurement

Cardiac tissue was procured from excess pathological tissue collected at Emory Hospital (Atlanta, GA) and from the Cooperative Human Tissue Network (Birmingham, AL). All autopsy cardiac tissue was obtained within 8 h after death and subjected to detailed pathological analysis.

DNA preparation

Cardiac muscle total DNA was prepared by sodium dodecyl sulfate-proteinase K digestion at 55°C and organic extraction (Sambrook et al., 1989).

PCR quantitation of mtDNA deletions

To estimate mtDNA damage, heart DNAs were serially diluted and each dilution tested using the polymerase chain reaction (PCR) for the presence of total and deleted mtDNA molecules. The percentage of deletions was calculated by dividing the dilution at which the deleted mtDNAs were lost by the dilution at which all mtDNAs were lost. The percentage of mtDNAs harboring the $mtDNA^{4977}$ deletion (np 8469–13,447) (Shoffner et al., 1989), the $mtDNA^{7436}$ deletion (np 8637–16,084) (Ozawa et al., 1990) and the $mtDNA^{10,422}$ deletion (np 4399–14,821) (Ballinger et al., 1992) were determined by PCR amplification across each deletion breakpoint. The $mtDNA^{4977}$ deletion was amplified using primers positioned at np 82,828–8305 and 13,851–13,832 (593 bp product), the $mtDNA^{7436}$ deletion was amplified using primers positioned at np 8150–8166 and 16,159–16,142 (575 bp product), and the $mtDNA^{10,422}$ deletion was amplified

TABLE 1

CONTROL HEART CHARACTERISTICS

Case	Age/Sex	Cardiac pathology	$mtDNA^{4977}$ deletion (%)
1	30/M	Left ventricular hypertrophy weight 490 g. Died of massive pulmonary hemorrhage secondary to Godpasture's syndrome	0.00001
2	39/M	Heart weight 370 g with epicardial hemorrhage	0.00022
3	63/F	Heart weight 650 g. Diffuse fibrosis in the heart overall shown to be edema and atrophy	0.00053
4	64/F	No evidence of myocardial ischemic damage or of atherosclerotic disease of coronary arteries. Hypertension caused heart failure and mitral regurgitation	0.006
5	64/M	Cardiac hypertrophy, right and left ventricles, weight 580 g. No evidence of myocyte necrosis, scattered lymphocytes	0.007
6	65/M	Atrial fibrillation, acute hemorrhagic infarction (3 days duration), myocardial hypertrophy, lipofuscin pigmentation and hypertension	0.0035
7	68/F	Hypertrophy and hypertension, left ventricle dilation. Degree of atherosclerosis extremely minimal	0.0045
8	74/F	Myocytes with hypertrophy, accumulation of neutrophils	0.0045
9	75/M	Heart weight 470 g, right ventricular dilation, left ventricular hypertrophy and interstitial fibrosis; lipofuscin pigmentation	0.0035
10	81/F	Hypertrophy biventricular, heart dilation, weight 610 g. Fibrosis posterior left ventricle, hypertension	0.0054

All the tissues analyzed were from autopsy hearts.

using primers positioned at 4308–4325 and 15,226–15,207 (496 bp product). The combined mtDNAs in each preparation were detected by PCR amplification within the rarely deleted region of the ND1 and 16S rRNA genes between positions np 3108–3127 and 3717–3701 (609 bp product).

Prior to amplification the sample DNA was digested with *Pst*I (np 6910 and 9020) and *Hin*dIII (np 6203, 11,680, and 12,567) which cut normal mtDNAs 3–5 times within the deleted regions. This avoided the amplification of wild-type products during the PCR reaction.

Polymerase chain reactions were performed as previously described (Shoffner et al., 1989) except that the first cycle had a 2-min denaturation (94°C), which was reduced to 30 s in the subsequent 34 cycles. Annealing was performed at 56°C for mtDNA^{4977} deletion, or at 51°C for total mtDNA, or the mtDNA^{7436} and mtDNA^{10,422} deletions. Serial dilutions of 2 μg of *Pst*I and *Hin*dIII digested DNA were performed to yield concentrations of 300 ng to 2.3 ng for the deleted mtDNA test and of 800 pg to 0.2 pg for the total mtDNA test. 90 μl of each PCR reaction was electrophoresed on 1.2% SeaKem agarose Tris–acetate–ethylenediaminetetraacetic acid (pH 7.6) gel containing 0.5 μg/ml ethidium bromide, and the DNA products were photographed under UV transillumination and analyzed by laser densitometry (Shoffner et al., 1990). The decline in PCR products for each dilution series was fitted to a sigmoid curve and the percentage of mtDNA deletions was calculated by the ratio of the DNA dilutions which reduced the PCR product optical densities of the deletion curve and the total mtDNA curve to the same levels (Corral-Debrinski et al., 1991).

To confirm each diagnostic PCR product, the fragments were digested with restriction endonucleases recognizing-sites on each side of the breakpoint. The mtDNA^{4977} deletion product was digested with *Hin*cII (np 13,664) or *Dde*I (np 8309 and 13,554), the mtDNA^{7436} deletion product was digested with *Rsa*I (np 16,096) or *Xba*I (np 8286) and the mtDNA^{10,422} deletion product was digested with *Xho*I (np 14,954) or *Dde*I (np 4356, 15,073 and 15,166).

A standard curve for the mtDNA^{4977} deletion was prepared using known mixtures of normal mtDNA and deleted mtDNAs (Corral-Debrinski et al., 1991). We have not prepared standard

TABLE 2

ATHEROSCLEROTIC HEART DISEASE CHARACTERISTICS

Case	Age/Sex	Cardiac pathology	mtDNA^{4977} deletion (%)
1	42/M	Severe atherosclerosis of coronary arteries, Cx 75%, LAD 75%, recent myocardial infarction, left ventricular hypertrophy	0.008
2	46/F	Congestive heart failure with cardiomegaly, wide range of ischemic changes, severe CAHD, old anterior infarction of endomyocardium	0.042
3	66/F	Severe CAHD, RCA over 85%, LAD 95%, Cx 95%, cardiomegaly and myocardial fibrosis	0.033
4	66/M	CAHD since 1983, graft to LAD and distal LC graft to distal RC 100% occluded. Native LC, LAD and RC at least 90% occluded, myocardium with chronic fibrosis	0.08
5	69/M	Severe CAHD, RCA over 95%, LAD 90%, Cx 70%, distal left main artery 40%, acute myocardial infarction and prolonged ischemia, occlusion-reperfusion injury	0.22

Tissue was procured from autopsy hearts. LAD, left anterior descending artery; Cx, circumflex artery; RCA, right coronary artery.

curves for the mtDNA^{7436} and mtDNA^{10,422} deletions. Hence, the reported levels are relative levels rather than absolute values.

ANT1 transcript analysis

Northern blots of OXPHOS gene transcripts were prepared using total cellular RNA (Torroni et al., 1990), and hybridized with an ANT1 cDNA probe (Neckelman et al., 1987). Relative ANT1 mRNA levels were estimated by laser densitometry scanning of autoradiographs, and normalization to rRNA levels determined by laser densitometric analysis of the ethidium bromide-stained 28S and 18S rRNA bands on the nylon filters.

Results

The mtDNA^{4977} deletion accumulates in older normal hearts and in CAHD

Ten pathological normal hearts, with no sign of cardiovascular disease, were obtained from subjects between ages 30 and 81 years (Table 1). Hearts from the 30 and 39 year old subjects had very low levels of the mtDNA^{4977} deletion: 0.0001% and 0.00022% respectively. Individuals older than 60 years had values ranging from 0.00053% to 0.007% of the total mtDNA, confirming that mtDNA damage increased with age.

Quantitation of the mtDNA^{4977} deletion in four chronic CAHD hearts (Table 2) revealed a 7–220-fold increase in the level of mtDNA damage relative to age-matched controls with values ranging from 0.033% to 0.22%. One patient died of acute myocardial infarction at 42 years of age. His heart contained 0.008% deletion, 42 times that of aged-matched controls.

Fig. 2 summarizes the results obtained for all the cases examined, including those previously reported (Corral-Debrinski et al., 1991). Two important conclusions can be drawn. First, in clinically normal hearts the mtDNA^{4977} deletion accumulates with age in individuals older than 30 years. Second, hearts experiencing chronic ischemia due to coronary artery disease accumulate much higher levels of the mtDNA^{4977} deletion than age-matched controls.

Multiple mtDNA deletions accumulate in normal older hearts and CAHD

To determine if other mtDNA mutations also accumulate in CAHD, we tested for the presence of two other mtDNA deletions, mtDNA^{7436} and mtDNA^{10,422}. Two patients with severe atherosclerosis were examined: a 49 year old patient who harbored 0.33% of the mtDNA^{4977} deletion, and a 56 year old patient who had 0.17% (Corral-Debrinski et al., 1991). The heart of the 56 year old patient contained about 0.15% of the mtDNA^{7436} deletion and about 0.1% of the mtDNA^{10,422} deletion, levels in the same range as the mtDNA^{4977} deletion (Fig. 3A), and extremely high compared to normal hearts (Fig. 3B). The heart from the 49 year old patient was found to harbor about 0.25% of the mtDNA^{7436} deletion and 0.15% of the mtDNA^{10,422} deletion, again comparable to the mtDNA^{4977} deletion (data not shown). The sum of the three types of mtDNA deletions was 0.78% for the 49 year old heart and 0.42% for the 56 year old heart. This confirms

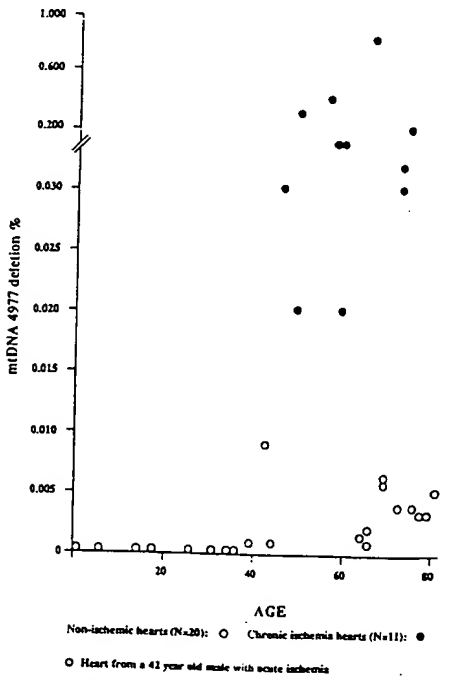

Fig. 2. The mtDNA^{4977} deletion vs. age for control hearts (bottom, open circles) and chronic coronary atherosclerotic hearts (top, closed circles).

that hearts with coronary artery disease contain a heterogeneous population of deleted mtDNA molecules.

To determine if pathologically normal hearts accumulate a similar array of mtDNA mutations, we tested for the three types of mtDNA deletions in hearts from normal individuals between 18 and 81 years of age (Fig. 3B). Hearts from individuals younger than 40 years (A, B, D and E) did not have detectable deletions, while hearts from individuals older than 40 years (C, F, G and H) had all three types of deletions. Thus, all three deletions accumulated in normal hearts with age as well.

In CAHD the left heart accumulates more mtDNA damage than the right heart

To determine if different regions of coronary atherosclerotic hearts accumulated mtDNA damage to different extents, we compared pairs of regions from various CAHD hearts. First, we compared the left ventricle and left atrium of patient 1 (Table 2). Here, the left ventricle accumulated 27 times (0.008%) more deletion than the left atrium (0.0003%) (Fig. 4A). Next, we compared the left and right ventricles of patient 4 (Table 2). The left ventricle accumulated 50 times more deletion (0.08%) that the right ventricle (0.0015%) (Fig. 4B). Finally, we compared the

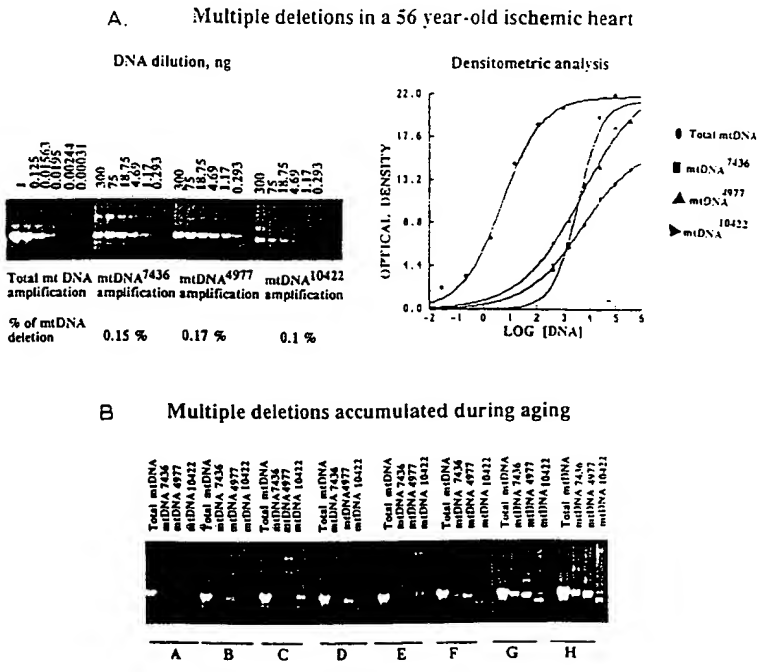

Fig. 3. (A) Left, PCR quantitation of mtDNA^{4977}, mtDNA^{7436}, and mtDNA$^{10.422}$ deletions in a 56 year old patient with severe coronary artery disease. Right, densitometric analysis of each PCR test. (B) Detection of the three types of deletions in non-atherosclerotic hearts of different ages. 300 pg of DNA was tested for the presence of all the mtDNA molecules (total mtDNA PCR test: lane 1) and 300 ng of DNA was tested for deletions: mtDNA^{7436} lane 2, mtDNA^{4977} lane 3 and mtDNA$^{10.422}$ lane 4. Samples are arranged as follows: A, 18 years old (case 4, Table 1, Corral-Debrinski et al., 1991); B, 30 years old (case 1, Table 1); C, 44 years old (case 8, Table 1, Corral-Debrinski et al., 1991); D, 39 years old (case 2, Table 1); E, 35 years old (case 7, Table 1, Corral-Debrinski et al., 1991); F, 63 years old (case 3, Table 1); G, 74 years old (case 8, Table 1); H, 81 years old (case 10, Table 1).

left and right atrium from a 58 year old patient (Table 2, case 3, Corral-Debrinski et al., 1991). The left atrium had 46 times more deletion (0.16%) than the right atrium (0.0035%) (Fig. 4C). These results demonstrate that mtDNA damage accumulates to different extents in different regions of the same organ. Therefore, it appears that mtDNA damage preferentially accumulates in the left heart over the right heart.

ANT1 transcript levels are elevated in CAHD and in left ventricle versus right ventricle

Previously, we observed that CAHD hearts accumulated high levels of nuclear and mitochondrial OXPHOS gene transcripts (Corral-Debrinski et al., 1991). This may be a compensatory response to mtDNA dysfunction and reduced ATP level. To determine if this phenomenon is also regional, we compared the ANT1 mRNA levels in the left and right ventricles of normal and CAHD hearts (Fig. 5).

Consistent with our previous study, the ANT1 mRNA levels in the left ventricle of CAHD hearts (cases 2, 3 and 4, Table 2; lanes 4, 5 and 1 of Fig. 5) was about 3.5-fold higher than a normal heart (case 2, Table 1; lane 3 of Fig. 5). Moreover, comparison of the left ventricle to the right ventricle of a CAHD heart (case 4, Table 2) revealed a 4-fold higher ANT1 mRNA level in the left ventricle (lanes 1 versus 2 in Fig. 5). Thus, like mtDNA damage, OXPHOS gene expression increases in CAHD and the induction is more apparent in the left ventricle.

Discussion

Accumulation of mtDNA damage during aging

Aging is a complex biological process associated with the age-related decline in stable organs such as brain, heart, muscle, kidney and liver. This has been associated with the progressive decline of OXPHOS enzymes in human skeletal muscle (Trounce et al., 1989) and liver (Yen et al., 1990), and the accumulation of mtDNA rearrangements in aging rodents (Piko et al., 1988). The accumulation of mtDNA damage with age has been proposed to be the cause of OXPHOS decline (Linnane et al., 1989), and it is thought to be mediated through oxygen radical damage (Miquel, 1986, 1991; Harman, 1983, 1986).

mtDNA damage has been shown to accumulate in human heart and brain tissues (Cortopassi and Arnheim, 1990; Hattori et al., 1991; Corral-Debrinski et al., 1991). In heart, the damage first

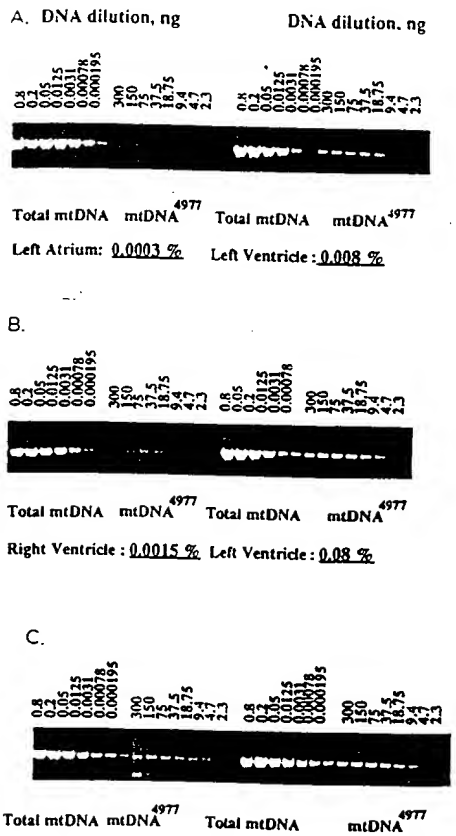

Fig. 4. Comparison of the amount of the mtDNA^{4977} deletion in different regions of CAHD hearts. Total mtDNA: total mtDNA PCR test; mtDNA^{4977}: mtDNA^{4977} deletion PCR test; values for the mtDNA^{4977} deletion in the different regions analyzed are expressed in percentage. (A) 42 year old patient with acute ischemia (case 1, Table 2); left atrium and left ventricle analyzed. (B) 69 year old patient with severe atherosclerosis (case 4, Table 2), right and left ventricles analyzed. (C) 58 year old patient with severe atherosclerosis; right and left atrium analyzed (case 3, Table 2, Corral-Debrinski et al., 1991).

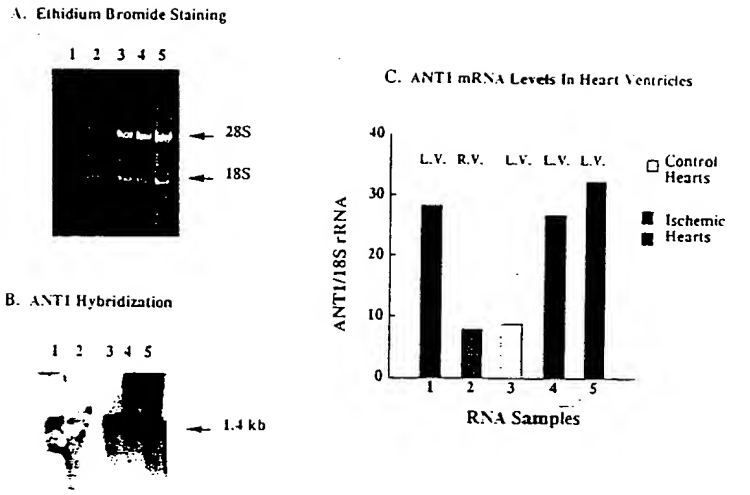

Fig. 5. ANT1 mRNA levels in CAHD hearts. (A) Negative of the ethidium bromide stained gel (see Methods). Lane 1, left ventricle from cardiovascular patient (case 4, Table 2); lane 2, right ventricle of the same patient; lane 3, control heart 2 (Table 1); lane 4, coronary atherosclerotic heart 2 (Table 2); lane 5, coronary atherosclerotic heart 5 (Table 2). (B) Northern blot autoradiograph hybridized with the ANT1 nuclear OXPHOS probe, on the left the apparent transcript molecular weight. (C) Quantitation of ANT1 mRNA levels. The values were determined by quantitative scanning laser densitometry of the filters before hybridization and the autoradiographs. Data are expressed in arbitrary units normalized by the relative intensity of the 18 S rRNA bands. L.V., left ventricle; R.V., right ventricle.

becomes apparent after age 35 (Cortopassi and Arnheim, 1990; Corral-Debrinski et al., 1991). mtDNA deletions accumulated along with mild age-associated physiological and structural changes of the heart (Fleg, 1986; Lakatta, 1987). This has been confirmed in the current study in which the mtDNA^{4977} deletion first appeared at age 39 and increased thereafter to a maximum of 0.007%. Moreover, aging hearts accumulate other forms of mtDNA damage as well, including the mtDNA^{7436} and mtDNA^{10,422} deletions, all of which appear about age 40. The accumulation of the mtDNA^{10,422} deletion is particularly significant since it lacks the light strand origin of replication and does not appear to have a replicative advantage in stable tissues (Ballinger et al., 1992). Therefore, the accumulation of these deletions must reflect random mutational events, rather than the progressive enrichment of a few deleted molecules, as occurs in CPEO and KS syndromes (Larsson et al., 1990).

The accumulation of mtDNA damage is accelerated in coronary artery heart disease

Atherosclerotic occlusion of the coronary arteries impedes the flow of nutrients and oxygen to the cardiac mitochondria. Reperfusion with oxygen rich blood creates oxygen radicals (Granger et al., 1981) and dramatically increases tissue damage (Guarnieri et al., 1980). The resulting chronic deprivation of the heart of mitochondrial energy ultimately can lead to cardiac overload and failure (Katz, 1989, 1990).

Consistent with this concept, both our previous study (Corral-Debrinski et al., 1991) and the current work have shown that CAHD is associated with dramatically elevated mtDNA damage and the concomitant induction of OXPHOS gene transcripts. While the mtDNA^{4977} deletion rose to a maximum of 0.007% in normal hearts it increased to between 0.02% and 0.85% in CAHD. Moreover, a heart which failed because of acute myocardial infarction contained less deleted

mtDNA than chronically CAHD but showed a 42-fold increase relative to age-matched controls. Thus ischemia/reperfusion causes mtDNA damage but prolonged ischemia/reperfusion is required to accumulate extensive damage.

The generalized nature of the mtDNA damage was confirmed by showing that the $mtDNA^{7436}$ and $mtDNA^{10,422}$ deletions accumulated in parallel wih the $mtDNA^{4977}$ deletion. This suggests that hearts with coronary atherosclerotic disease harbor a broad spectrum of deleted mtDNAs.

Regional accumulation of damage

Since the left heart has a higher work load and energy requirement than the right heart. ischemia would be expected to produce a greater energy depletion and oxygen radical generation in that region. Consistent with that hypothesis, the left ventricle of CAHD hearts was found to accumulate 50-fold more mtDNA damage than the right ventricle and 27-fold more damage than the left atrium. Moreover. ANT1 mRNA was induced 4-fold more in the left ventricle of a CADH heart than the right ventricle. These observations support the hypothesis that increased energy demands (cardiac work load) and free radical production are important in the accumulation of mitochondrial damage.

Thus mtDNA damage does increase with age in stable tissue, suggesting that mtDNA damage may be an underlying cause for organ failure in aging. Further, energetic overload increases the rate at which the damage accumulates, but the overload can only partially be offset by induction of OXPHOS gene expression. These phenomena could explain why patients with inherited defects in OXPHOS have late onset of symptoms and a progressive clinical course.

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EXHIBIT 2

ELSEVIER

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BIOCHIMICA ET BIOPHYSICA ACTA
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Role of mitochondria in oxidative stress and ageing

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Abstract

Mitochondria are deeply involved in the production of reactive oxygen species through one-electron carriers in the respiratory chain; mitochondrial structures are also very susceptible to oxidative stress as evidenced by massive information on lipid peroxidation, protein oxidation, and mitochondrial DNA (mtDNA) mutations. Oxidative stress can induce apoptotic death, and mitochondria have a central role in this and other types of apoptosis, since cytochrome *c* release in the cytoplasm and opening of the permeability transition pore are important events in the apoptotic cascade. The discovery that mtDNA mutations are at the basis of a number of human pathologies has profound implications: maternal inheritance of mtDNA is the basis of hereditary mitochondrial cytopathies; accumulation of somatic mutations of mtDNA with age has represented the basis of the mitochondrial theory of ageing, by which a vicious circle is established of mtDNA damage, altered oxidative phosphorylation and overproduction of reactive oxygen species. Experimental evidence of respiratory chain defects and of accumulation of multiple mtDNA deletions with ageing is in accordance with the mitochondrial theory, although some other experimental findings are not directly ascribable to its postulates.

Keywords: Mitochondrion; Oxidative stress; Mitochondrial DNA; Aging

1. Introduction

After the elucidation of the major aspects of energy conservation in oxidative phosphorylation, the declined interest in mitochondria received a novel impetus by discoveries of cell biology and pathology. Two major developments opened breakthroughs in mitochondrial pathology: first, the discovery that mitochondrial DNA (mtDNA) mutations are at the basis of diseases [1], and second, the unexpected role of mitochondria in the mechanisms of cell death [2]. A common denominator of these aspects is the role of reactive oxygen species (ROS). This article attempts to provide a rationale for the role of oxidative stress by ROS on different aspects of pathology where mitochondria seem to have a major role. There is almost no area of human pathology where oxidative stress has not been implicated [3,4]; this review, therefore, will be restricted to few selected topics and will be centered on the role of mitochondria in ageing.

Abbreviations: CoQ_n, coenzyme Q with *n* isoprenoid units; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial enkephalomyopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonal epilepsy with ragged-red fibers; mtDNA, mitochondrial DNA; NARP, neurogenic muscle weakness, ataxia and retinitis pigmentosa; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction; PEO, paralysis of the extraocular muscles; ROS, reactive oxygen species

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2. Mitochondria as sources of ROS

Reactive oxygen species [5,6] include oxygen free radicals (the superoxide radical anion, as the primary product of one-electron dioxygen reduction [7,8], and the extremely aggressive hydroxyl radical deriving from subsequent chemical reactions [9]), singlet oxygen and strong non-radical oxidants such as hydrogen peroxide; furthermore nitric oxide and the derived peroxynitrite radical [10] can be included in this category.

Sources of ROS in living cells are represented by physiological enzymatic mechanisms [11]; oxidative stress [11] may ensue when ROS production is excessive, due either to a particular metabolic situation, or to the presence of xenobiotic compounds [12], or also to damage-mediated liberation of non-enzymatic catalysts such as free metals [13,14], or when the cellular defences are lowered by the depletion of physiological antioxidants.

Among the cellular sources of ROS, besides specific enzyme systems involved in phagocytosis [8], eicosanoid metabolism [15] and nitric oxide production [16], are cytoplasmic systems such as xanthine oxidase [4], that can be formed from xanthine dehydrogenase by oxidation of thiol groups [17], microsomal P_{450} oxygenases and quinone reductases [18], and the mitochondrial respiratory chain [19].

The respiratory chain is a powerful source of ROS, primarily the superoxide radical and consequently hydrogen peroxide, either as a product of superoxide dismutase [7] or by spontaneous disproportionation. It is calculated that 1–4% of oxygen reacting with the respiratory chain is incompletely reduced to ROS [20,21]. Their production may increase in state 4 with respect to state 3 [22], because O_2 concentration increases and the level of reduced one-electron donors in the respiratory chain is concomitantly increased [23]. A similar result is obtained when cytochrome oxidase activity is lowered, since this leads to the concomitant raise of oxygen concentration and of the reduced state of one-electron donors in the respiratory chain [24]. According to Skulachev [25], uncoupled electron transfer chains, by enhancing oxygen consumption, represent a device for preventing or decreasing ROS production by mitochondria.

There are two major respiratory chain regions where ROS are produced, one being complex I (NADH coenzyme Q reductase) [8,20,26–28] and the other complex III (ubiquinol cytochrome *c* reductase) [8,28–30].

In complex III, antimycin is known not to completely inhibit electron flow from ubiquinol to cytochrome *c*: the antimycin-insensitive reduction of cytochrome *c* is mediated by superoxide radicals; the source of superoxide in the enzyme may be either cytochrome b_{566}, [31] or ubisemiquinone [32] or Rieske's iron-sulfur center [33]. Ubisemiquinone is relatively stable only when protein bound [34], therefore the coenzyme Q (CoQ) pool in the lipid bilayer is no source of ROS.

The role of ubiquinone within ROS production deserves some comments (cf. [18]), since it has been described both as a prooxidant [22,28,32] and as a powerful antioxidant [35–37]; the former action has been ascribed to either oxidized or reduced quinone, whereas the latter exclusively to ubiquinol.

In some instances, a prooxidant effect may be ascribed only apparently to CoQ: for example the enhanced ROS production when CoQ-depleted mitochondria oxidizing succinate are reconstituted with CoQ [22] is a consequence of the increased rate of electron feeding to complex III via the quinone, and presumably complex III itself is the source of ROS generation.

Early experiments proved the involvement of complex I in ROS production [38]; addition of either NADH at low concentration or of NADPH, which feeds the electrons at decreased rate into the complex, led to copious ROS production detected by lipid peroxidation; on the other hand, addition of NADH at high concentration, but in the presence of rotenone, also induced peroxidation. In another study [28] water-soluble CoQ homologs used as electron acceptors from isolated complex I stimulated H_2O_2 production in the order $CoQ_1 > CoQ_0 > CoQ_2$, whereas CoQ_6 and CoQ_{10} were inactive; the rate of H_2O_2 production was partly inhibited by rotenone, indicating that water-soluble quinones may react with oxygen when reduced at sites both prior and subsequent to the rotenone block. There is evidence that the one-electron donor to oxygen in complex I is a non-physiological quinone reduction site different from the physiological site(s) [39–41]; the former, hydrophilic, site reduces several quinones to the corresponding semiqui-

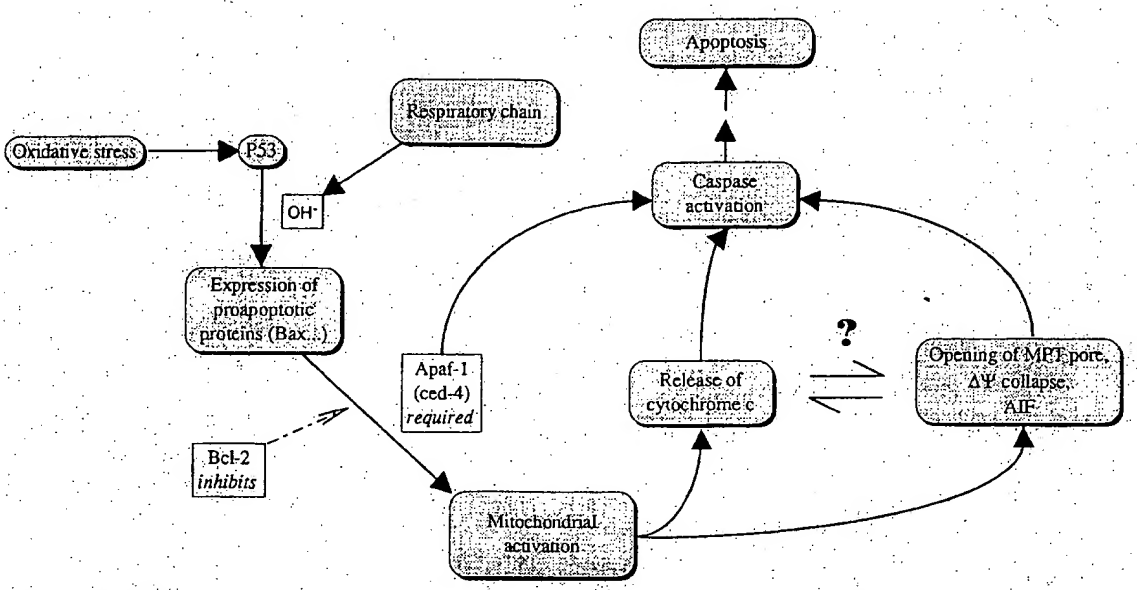

Fig. 1. A scheme of oxidative stress-induced apoptosis (cf. [113,159]). See text for explanations. MPT, mitochondrial permeability transition, AIF, apoptosis inducing factor. Mitochondrial activation refers to an early unknown step (steps) within the bulk of phenomena occurring at the mitochondrial level in the apoptotic cascade.

none forms, which are unstable and can reduce oxygen to superoxide. This mechanism is shared by several quinones, including such drugs as anthracyclines [42] and the clinically employed CoQ analog, idebenone [43]. However, auto-oxidation of fully reduced quinones [18], such as those formed by NADH CoQ reductase past the rotenone inhibition site, is also a source of ROS, but this effect exclusively pertains to hydrophilic quinones, and not to the physiological hydrophobic ubiquinol. Finally, in view of the experiments of Takeshige et al. [38], the hydrophilic, rotenone-insensitive, site can apparently reduce oxygen to superoxide in the absence of intermediate acceptors.

To conclude about the role of CoQ, it is clear that hydrophobic ubiquinones, such as the physiological CoQ_{10} for man, do not behave as prooxidants in mitochondria; on the contrary, all evidence points out that they behave as antioxidants in their reduced form [35–37], possibly, their deep membrane insertion prevents contact with non-physiological reduction sites, and they are not auto-oxidizable. The mechanism by which reduced CoQ functions as an antioxidant in vivo is out of the scope of this review and is discussed at length elsewhere [35–37].

ROS production by the respiratory chain is increased after a period of anoxia, when the oxygen concentration is reestablished by reperfusion [44–46]; one reason, besides those pointed out above (sudden high oxygen concentration in the presence of the respiratory chain in the reduced state), may be that the anoxic period, by depleting ATP, has induced damage to cellular structures and released catalytic metals [47], such as iron and copper, which are abundant in the inner mitochondrial membrane.

Mitochondria contain antioxidant enzymes, including superoxide dismutase (Mn form) [48,49] and glutathione peroxidase [50], and lipid-soluble antioxidants such as vitamin E [51] and reduced CoQ [52]. Ubiquinol may exert its antioxidant function indirectly by reducing α-tocopheroxyl radical back to vitamin E [53] or directly as a quencher of oxygen and lipid peroxyl radicals [54,55].

3. Mitochondria as targets of ROS

Being major producers of ROS, mitochondrial structures are exposed to high concentrations thereof and may therefore be particularly susceptible to their attack. Evidence exists, however, that even ROS produced outside the mitochondrion may damage mito-

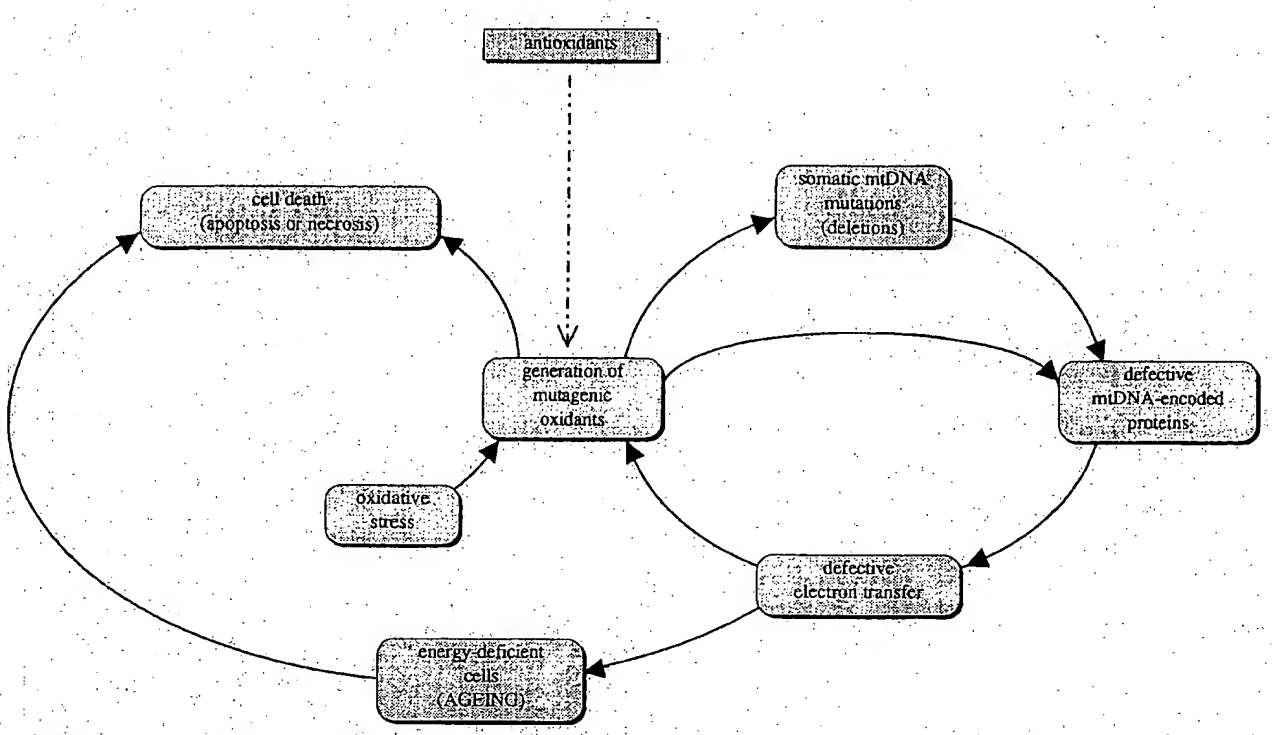

Fig. 2. A vicious circle of oxidative stress and mtDNA mutations in cell death and ageing (freely redrawn from [159,218]).

chondrial components: decreases of respiratory enzyme activities and of mitochondrial membrane potential, induced by adriamycin in perfused rat liver [56] or isolated hepatocytes [57], are prevented by incubation with CoQ_{10}; the exogenous quinone is taken up by the intracellular compartment but is not incorporated in the mitochondria under the conditions of the experiments [58], suggesting that the radicals quenched by CoQ are also produced in the extramitochondrial compartment.

Damage by oxidative stress to mitochondrial components includes lipid peroxidation, protein oxidation and mtDNA mutations.

Lipid peroxidation [14,59–61] might be particularly harmful in mitochondria, that contain cardiolipin as a major component of the inner mitochondrial membrane [62], since this lipid is required for the activity of cytochrome oxidase [63] and of other mitochondrial proteins [64]. Oxidative stress decreases cardiolipin to a larger extent than other lipids [65,66], perhaps as a consequence of its high unsaturation [67]; cardiolipin decrease appears to be directly related to reversible decrease of cytochrome oxidase activity [68,69].

Protein oxidation as a result of oxidative stress may occur either directly or as a consequence of lipid peroxidation [70–73]; it has been described to affect respiratory chain enzymes [74], ATPase [73–76], the adenine nucleotide translocator [77] and transhydrogenase [78] and to determine opening of the permeability transition pore [79]. To this respect, it is worth emphasizing the striking susceptibility of the adenine nucleotide translocator to oxidative stress [77], since this protein is considered part of the permeability transition pore [80] (cf. [81] for review). Complex I is particularly susceptible to oxidative damage [82], and its decreased activity in Parkinson's disease may be linked to an enhanced production of ROS and to the consequent damage of the complex [83]. Modification of the redox state of vital sulfhydryl groups may be at the basis also in mitochondria of important regulatory mechanisms, similar to those suggested to modulate signal transduction cascades [84]. Inactivation of Mn-superoxide dismutase in transgenic mice [85] enhances ROS production and results in animal death by dilated cardiomyopathy, with partial inactivation of mitochondrial enzymes containing iron-sulfur centers.

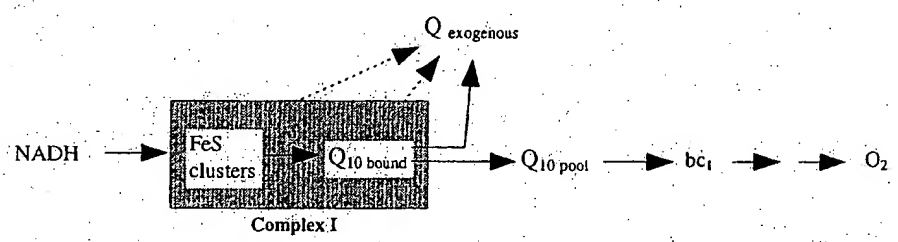

Fig. 3. A decreased electron transfer to oxygen via the CoQ pool may be masked, when assaying complex I activity, by concomitant reduction of the exogenous quinone acceptor by electrons flowing through both the physiological site and a non-physiological site (cf. [40]). A decreased electron flow to oxygen might depend on a decreased V_{max} of NADH-CoQ_{10} reductase activity of complex I, but also on its increased K_m for CoQ_{10} in the pool. In fact such a higher K_m may be responsible for decreased electron flow to oxygen, since the physiological CoQ_{10} concentration in the inner mitochondrial membrane is not saturating for NADH oxidation [176].

Of special interest is damage to mtDNA [86–89], since this small molecule is extremely susceptible to oxidative damage: being located in the matrix, it is close to the major source of ROS; moreover, lacking introns and being devoid of histones and other DNA-associated proteins, the probability of oxidative modification of a coding region of mtDNA is very high. After an oxidative stress to cultured cells, the damage to mtDNA is higher and persists longer than that to nuclear DNA [90], indicating that the repair devices of the former, even if present, are largely insufficient to overcome extensive DNA damage [91].

ROS were shown to induce extensive fragmentation and deletions in mtDNA; up to 187 different deletions were listed by Ozawa after the third day of an oxidative stress to a transformed human fibroblast cell line [92]. The 8-hydroxy-D-guanosine content is considered a marker of oxidative damage to DNA [93]; its increased content in mtDNA has been reported in ageing [93–95] and age-associated diseases [96–98].

4. Mitochondria, ROS, and cell death

Cell death can occur by either necrosis or apoptosis [99] as a result of exogenous and endogenous insults. There seems to be no net border between these phenomena [100], depending mainly on the extent of stress [101] and on the ATP levels [102]; however, the mechanisms are rather different, since apoptosis involves a well defined chain of enzymatic events which are genetically programmed [103]. Apoptosis induced by oxidative stress has been well documented [92,101,104–106] and appears to involve the same steps in the commitment and execution stages as in the other causes of apoptosis [105,106]. Actually, apoptosis may be a mechanism to eliminate ROS-producing cells [107].

A recent exciting development has been the discovery that there is an early mitochondrial involvement in the apoptotic cascade [103,106] (Fig. 1). The release of cytochrome *c* from the intermembrane space has been reported as an early event activating the product of a gene (corresponding to *ced-3* in the nomenclature of the activation sequence of the nematode *Caenorhabditis elegans*), which is *caspase-3* (a cysteine-aspartate protease) [108–111]; this activation requires the product of the *ced-4* gene [112] and is inhibited by *ced-9* or Bcl-2 and similar proteins [110,111,113–115]. The proteins of the Bcl-2 family are localized in the outer mitochondrial membrane [116], where pro-apoptotic proteins such as Bax and Bad, structurally related to the former, are also present [117]. Since opening of the permeability transition pore, with fall of the mitochondrial membrane potential, also represents a fundamental step in apoptosis [103,106,118], and Bcl-2 may also prevent pore opening [119], the relation between cytochrome *c* release and pore opening is still controversial: whether the latter phenomenon, with consequent swelling and rupture of the outer membrane, is the cause of cytochrome *c* release, as it happens in isolated mitochondria [120,121], whether cytochrome *c* loss and consequent alteration of the respiratory chain induce pore opening, or whether they are two independent phenomena. The fact that Bcl-2 and related proteins can polymerize [122] to form large channels in the membrane [123,124] has suggested

that such channels in the outer membrane may allow the release of cytochrome *c* in the cytosol [117,123].

Oxidative stress may not just be *one* out of several signals committing the cell to apoptosis, but represent an early intrinsic component of any apoptotic cascade, since an oxidative stress component was shown in apoptosis induced, among others, by p-53 expression [125], by dexamethasone [126], and by ceramide [127]; in the latter, the oxidative step was identified in complex I of the respiratory chain [127].

Although oxidative stress is one of the causes of pore opening [79], the commitment to apoptosis seems to be an earlier event, at least in some forms of apoptosis [128]: induction of pro-apoptotic proteins and cytochrome *c* release seem to occur before any decrease of the mitochondrial transmembrane potential can be shown [110,111].

5. Mitochondrial cytopathies as a model for ageing

The discovery that mtDNA mutations are at the basis of a number of human pathologies [129] has opened a new and extremely active chapter of mitochondrial research [130–132]. Not all mitochondrial cytopathies are due to mtDNA mutations, but many are due to mutations in nuclear genes encoding for mitochondrial proteins [133,134]. The diseases due to mtDNA mutations, contrary to those of nuclear genes, are genetically well characterized. There are diseases due to point mutations of a structural gene and others due to mutations in tRNA genes; in addition sporadic or familiar forms characterized by extensive deletions of mtDNA are also described [129–134].

Mitochondrial genetics is peculiar for many respects when compared with mendelian genetics [132,134]: besides the maternal type of transmission, the presence of multiple mtDNA copies in the same cell (polyplasmy) and the possibility for a mutation to experience different degrees of heteroplasmy are at the basis of the threshold effect, by which a phenotypic lesion becomes evident only when over 80% of the mtDNA in a cell is mutated, and of mitotic segregation, by which the proportion of mutant mitochondrial genomes may shift in daughter cells during cell division; surprisingly, the process appears to be non-random, so that different genotypes segregate to different extents in different tissues during embryogenesis [135,136], a finding that can have strong relevance to ageing (Section 6).

Mitochondrial structural genes encode for 13 polypeptide chains belonging to those complexes of the inner mitochondrial membrane which are involved in the transmembrane movement of protons. Thus, the phenotypic consequence of a mtDNA mutation must be a defect in the oxidative phosphorylation machinery of mitochondria [129].

A lesion in a structural gene, such as due to a point mutation, would interfere with the function of the polypeptide encoded by that gene, leading to enzyme activity decrease. Indeed this is often found in such kinds of cytopathies, such as a form of Leber's hereditary optic neuropathy (LHON), characterized by a mutation in the genes for the ND1 subunit of complex I at position 3460 of mtDNA [137], but not in the form harboring the 11 778 ND4 mutation, where complex I activity is unchanged [138]; in spite of normal complex I activity, the flux over the entire respiratory chain is significantly decreased [138].

The finding that in the 11 778 ND4 mutation there is a decreased sensitivity to rotenone [139] and a decreased affinity for a quinone homolog used as the acceptor [139,140] agrees with the notion that the seven ND subunits of complex I are hydrophobic polypeptides belonging to the sector of the enzyme where a large series of inhibitors and the electron acceptor CoQ are bound [141]. Since the quinone-binding sites are also involved in the proton translocation activity of the complex [141,142], it is predictable that a defective energy conservation may be present in this form of the disease.

The decreased respiratory rate in spite of normal complex I activity in subjects harboring the 11 778 ND4 mutation is puzzling; possible explanations are either in a decreased affinity of complex I for endogenous ubiquinone (CoQ_{10}), as indeed found for an exogenous short CoQ homolog, CoQ_2 [139,140], or in overestimation of the physiological complex I activity due to electron withdrawal, by the acceptor used, from a non-physiological site in the enzyme [40,43], made available by an incorrect assembly caused by the mutation [139].

Another cytopathy characterized by a mtDNA point mutation, the NARP or maternally inherited Leigh syndrome, associated with an 8993 mutation in

the ATPase-6 gene [143], is characterized by decreased ATP synthesis [144], although the biochemical defect has not yet been identified; in the light of the above considerations, it would be speculated that the primary defect involves proton translocation through the F_0 proton channel, as found by site-directed mutagenesis to the homologous region of subunit a of *Escherichia coli* [145].

On the other hand, a lesion in a gene encoding for a mitochondrial tRNA, as in the MELAS and MERRF syndromes [131], would interfere with mitochondrial protein synthesis of all the subunits encoded by mtDNA.

Some forms of mitochondrial disease are caused by mtDNA deletions [131]; being extended to large portions of the mitochondrial genome, as the 4977 bp common deletion found in patients affected by the Kearns-Sayre syndrome [146] together with many other deletions, and removing many genes including essential tRNA genes [147], they strongly interfere with mitochondrial function.

It was found that some mtDNA alterations, particularly deletions, are also present in normal individuals, though to very small extents, and accumulate with age [148]. In particular, the common deletion of the Kearns-Sayre syndrome has often been found by PCR to accumulate in postmitotic cells in old individuals.

6. Mitochondria and ageing

The concept that mitochondria are primarily involved in ageing derives from the theory of Harman [149], linking senescence to the injurious effect of free radicals arising from the one-electron reduction of oxygen during metabolism. In accordance with the free radical theory of ageing is the inverse relation existing between auto-oxidation rate in different animal species and life expectancy of the same species [150,151]; the auto-oxidation rate on its hand is directly proportional to metabolic rate, so that the duration of life seems to be inversely related to the rate of oxygen consumption [152]. The increased longevity obtained by caloric restriction in rodents [153,154], which is accompanied by decreased state 4 respiration and decreased superoxide production [154], and the relation of lifespan in *Drosophila* with the simultaneous expression of the antioxidant enzymes superoxide dismutase and catalase [155] are corollaries of this proposal.

6.1. Mitochondrial theories of ageing

As pointed out in Sections 2 and 3, mitochondria are the major sources of oxygen radicals through the respiratory chain and are also deeply affected by ROS, resulting in serious risks to their function. Indeed, a decreased mitochondrial performance has been generally observed in senescence (cf. [156]) and, in principle, this decline could be due to alteration of each one of the macromolecular components above. The mitochondrial theory of ageing [157,158] has attempted to define in precise molecular terms the energetic changes accompanying senescence, and can be represented as a refinement of the free radical theory.

Briefly, it was proposed that accumulation of somatic mutations of mtDNA, induced by continued exposure to free radical attack, leads to errors in the mtDNA-encoded polypeptide chains; these errors are stochastic and randomly transmitted during mitochondrial division and cell division. The consequence of these alterations, which affect exclusively the four mitochondrial enzymatic complexes involved in energy conservation, would be defective electron transfer and oxidative phosphorylation. In addition, respiratory chain defects may become associated with increased production of ROS, thus establishing a vicious circle of mtDNA mutations and oxidative stress [159]; the redox mechanism of ageing [159,160] unifies both the ideas of the mitochondrial theory [157,158] and of the free radical theory of ageing [149] (Fig. 2). It is also established that oxidative stress is a powerful cause of apoptotic death (Section 4); therefore oxidative stress, ageing and apoptosis are strictly linked events. It is conceivable that an acute stress would trigger a mechanism inducing cell death directly, whereas a milder stress may slowly lead to impaired cell function as in ageing.

The mtDNA mutations are expected to accumulate and to lead to damage mainly in postmitotic cells [161], where oxidative metabolism is very elevated as in neurons or is subjected to high bursts as in skeletal muscle; moreover, in postmitotic cells, the lesions

could be conserved at difference with mitotic cells where division leads to selection with 'washing away' of the defective cells [162].

6.2. Mitochondrial bioenergetics in ageing

The subject of age-dependent changes in mitochondrial bioenergetics abounds in conflicting data, e.g. reporting declines of respiratory enzymes or ATPase activity or unable to find significant differences (cf. [157,163]). There may be several reasons for such conflicting data.

First, mitochondria when isolated are obtained from tissues containing both differentiated non-dividing cells and relatively non-ageing dividing cells; thus small changes only in one population may become undetectable [161]. Furthermore, energy-defective cells may undergo elimination by apoptosis [164]; the continuous cell elimination when mitochondria become deficient would prevent observing important energetic changes in the remaining population.

If the energetic impairment derives from a stochastic damage to the mitochondrial genes, then it is important to select the mitochondrial activity which is most likely to be affected. Since seven out of the 13 structural genes in mtDNA encode for polypeptides in complex I (NADH CoQ reductase), then it is complex I that is most likely to undergo functional alterations [165]. Unfortunately the assay of complex I activity suffers from serious problems due to the choice of the best quinone to be used as electron acceptor [166]; in our laboratory it was found useful, when possible, to assay this activity indirectly [167] by exploiting the pool equation [168]

$$V_{obs} = (V_o \times V_r)/(V_o + V_r)$$

whereby the rate of CoQ reduction (V_r) is related to total rate of NADH oxidation by oxygen (V_{obs}) and to rate of ubiquinol oxidation (V_o). Using this method, significant decreases of NADH CoQ reductase activity, undetected by the direct assay, were revealed in liver and heart mitochondria from 24-month-old rats [167], presumably by providing more accurate values of NADH CoQ reductase activity.

Another approach employed in our laboratory for recognition of possible early changes not only in postmitotic cells but also in short-living cells, such as blood platelets, has been looking for specific changes linked to subunits encoded for by mtDNA; in analogy with previous findings in LHON [139], it was found that rotenone sensitivity of NADH CoQ reductase was significantly decreased in platelets from old individuals [169]. The same change was exhibited by non-synaptic mitochondria from rat brain cortex [170].

A decrease of an individual enzyme activity in a metabolic pathway is meaningful only if it is able to affect the rate of the whole pathway, and this will depend on the degree of flux control exerted by the individual step [171]: in the respiratory chain, complex I is present in lowest amounts [62], then it is presumably the rate-limiting step of aerobic NADH oxidation [172]; however, this is not true for the oxidation of NAD-linked substrates in phosphorylating mitochondria [173,174]. In mitochondrial diseases, the flux control coefficient at site I in permeabilized cells was found to dramatically increase [175]; unfortunately this point has not been addressed in studies on ageing.

The opposite phenomenon, i.e. the mentioned decreased respiratory rate accompanied by unchanged complex I activity, detected in livers and hearts of aged rats [167], was ascribed to inadequacy of commonly used acceptors; in analogy with a similar behavior in the LHON 11 778 ND4 mutation, an incorrect assembly of the complex, witnessed by a decreased rotenone sensitivity of its activity [169,170], and leading to artifactual pathways of electron transfer to short chain quinones [40,43], might explain the observation in molecular terms (Fig. 3). Alternatively, a decreased affinity for endogenous CoQ might explain this apparent paradox, since CoQ concentration is not saturating for NADH oxidation [176]. The latter possibility has not been explored in ageing; in view of the analogous finding of decreased rotenone sensitivity in ageing as well as in LHON mutations, where the K_m for a CoQ homolog is also increased [139], this point is obviously worth investigation.

Additional evidence on a respiratory impairment in senescence concerns histochemical detection of a loss of cytochrome *c* oxidase activity (but not of succinate dehydrogenase) in muscle mitochondria from old individuals [177]. The mosaic distribution of the cytochrome oxidase lesions is well in agreement with the stochastic distribution of mitochon-

drial damage predicted by the mitochondrial theory [164].

6.3. Mutations and deletions of mtDNA

Point mutations in mtDNA are present in normal individuals and were claimed to increase with age [178,179]; a careful study by quantitative PCR of the frequency in skeletal muscle of two point mutations observed in mitochondrial diseases has revealed that the mutations are present at very low percentage, but there is no correlation with age, at least up to 70 years [180].

The matter is different with mtDNA deletions. The so-called common deletion, observed to high extents in some mitochondrial disorders, was also found to increase exponentially with ageing, particularly in muscle and brain tissues [148]. Careful quantitation by a competitive PCR method revealed that this single deleted species was present in aged muscle at the level of only 0.1% of total mtDNA. Thus, it may be questioned whether detection of deleted mtDNA in the elderly only represents an epiphenomenon of the primary pathogenetic event, since very high levels of damage are necessary to elicit activity changes, in accordance with the threshold effect. It has to be borne in mind, however, that other deletions have been found to increase in ageing [148]. Thus, even though any single species of deleted mtDNA is present at low levels, the total amount of deleted mtDNA may reach levels that could be significant in terms of oxidative phosphorylation decline. Ozawa, by systematic use of PCR primers over the entire mDNA, found a progressive age-related fragmentation of mtDNA into various size deleted molecules up to 358 types, with a strong correlation with 8-hydroxy-D-guanosine accumulation [92,96,181]; mtDNA fragmentation also occurred in premature ageing [182]. Deleted forms of mtDNA lacking replication origins accumulated up to 280 types out of 358. Concomitantly, wild type mtDNA decreased down to 11% of total. Similar fragmentation of mtDNA [183,184] and extensive oxygen damage to tissues [185] was demonstrated in patients with mitochondrial cardiomyopathy, harboring hazardous germ-line point mutations; therefore the mitochondrial diseases seemed to be derived from premature ageing of the tissues.

A proof of the existence of a vicious circle of oxidation and mtDNA damage [159] requires demonstration that ROS production and their effects *increase* in ageing. It was indeed shown that hydrogen peroxide generation increases with age, e.g. in different species of flies [151] and in isolated rat hepatocytes ([186,187]; Cavazzoni and Lenaz, unpublished observation), although peroxide overproduction in ageing was not found by others (cf. [188]). Increased production of ROS is also inferred from the increase of oxidized cellular components with age [3,4,72,160], although this accumulation may also result from impaired disposal of the damaged species (cf. [189]).

According to the above view, the accumulation of mtDNA lesions with ageing derives from an unbalance in the rate of generation of ROS and their removal [159,160]; an alternative or additional explanation is that there is a relatively constant rate of generation of the mutations, leading to synthesis of aberrant proteins, but that in young animals cells exhibiting those mutations are eliminated by immunosurveillance by cytotoxic T-lymphocytes; since the protective immune response declines with age, leading to an altered repertoire of antigens recognized [190], one would expect an accumulation of cells containing altered mtDNA (H. Baum, personal communication). In the light of the above hypothesis, the decline in immunosurveillance with ageing may also well reflect somatic mutations of lymphocyte mtDNA (cf. [191]).

The significance of mtDNA deletions in ageing is emphasized by the species specificity of their time of arousal: mtDNA deletions accumulate in organisms with life spans significantly shorter than those of humans, at ages when there is no detectable deletion in humans [192]. To this purpose, when using rodents as experimental models for human ageing, the appropriate tissues should be considered, since not all tissues of rats accumulate mtDNA deletions in the same manner as those of humans [193].

6.4. Mitochondria-nucleus interplay in ageing

The available evidence suggests that mtDNA deletions accumulate to sufficient extents to be able to represent the cause for the respiratory chain and oxidative phosphorylation defects observed in ageing

[148]; the proof that this is indeed the case, however, is still lacking. The reason why deletions but, perhaps, not point mutations accumulate in ageing is not clear, although it may depend on the DNA repair capabilities of mitochondria [194]. Oxidative stress can lead to mtDNA deletions [92,94,95], although these can also arise from altered nuclear-cytoplasmic interactions. In fact an autosomal dominant form of PEO (paralysis of extraocular muscles), characterized by multiple mtDNA deletions, indicates that it is a nuclear gene product that affects the proclivity of mtDNA to suffer deletions [195]. Whether this is also the case for mtDNA deletions occurring in ageing is not known. The observation that oxidative phosphorylation defects in cultured cells from aged donors could be reversed by constructing cybrids where the nucleus from the old donor was substituted by a nucleus from an immortalized cell suggested that a nuclear mutation might be responsible for the mitochondrial deficit [196]; a subsequent extended study [197], however, demonstrated the formation of several respiratory-deficient clones by fusing mitochondria from fibroblasts of aged donors with a mtDNA-less ρ^0 cell line, as well as a significant decrease of respiration rate and of mtDNA content with the age of the donors. The results of the previous study [196] were ascribed to selection of respiratory competent clones during fusion.

Some additional observations on mitochondrial changes in ageing appear difficult to be directly explained by the mitochondrial theory. The decreased mtDNA transcription in heart and brain of aged rats [198] and the decreased rate of mitochondrial protein synthesis in human muscle [199] have suggested the existence of some more subtle defect in the interplay between nucleus and mitochondria. However, the findings that deleted mtDNA often lacks replication origins [159] and that the deletions usually encompass several tRNA genes may offer explanations in line with the mitochondrial theory. The suggestion that mitochondria in aged cells may increase in size [161,187,200,201] would also be in line with this explanation, as a sign of impaired mitochondrial division [160,161].

The dramatic changes in lipid composition observed in ageing, particularly the fall in cardiolipin content [202] and its relation with cytochrome oxidase activity [68,69], suggest that other factors, related to oxidative stress but not involving mtDNA, may be important pathogenetic events in the ageing process. The finding that a decreased rotenone sensitivity of NADH CoQ reductase is observed in platelets from old individuals [169], but that the 'common deletion' is not observed in mtDNA from the same cells [203], in contrast with a previous study [204], throws some uncertainty over the mtDNA damage as the *only* source of bioenergetic defect. Clearly, a mitochondrial involvement in ageing is present, though a unique role of mtDNA has yet to be established.

7. *Mitochondria and age-related diseases*

The possibility that some of the most common and devastating degenerative diseases of old age have a mitochondrial involvement has been seriously considered in recent years [205–207]. Evidence pertaining to this point will be only briefly summarized here. In particular an impairment to energy metabolism due to progressive failure of the mitochondria has been invoked in the pathogenesis of Alzheimer's disease, Huntington's disease, Parkinson's disease, and cerebellar degenerations. A common denominator of these diseases, affecting different specific brain areas with cellular atrophy, may be a reduced oxidative metabolism and ATP synthesis, leading to membrane depolarization with consequent activation of the glutamate NMDA channels and excitotoxic cell death [206], similar to the acute death produced by glutamate release in ischemia-reperfusion injury [208]. The observation that mitochondrial dysfunction in nervous tissue induces excitotoxic responses agrees with this hypothesis [209].

In Alzheimer's disease, the affected brain areas exhibit an increased production of 8-hydroxy-2-D-guanosine from mtDNA with respect to age-paired controls [96–98], an indication of enhanced oxidative stress in the diseased brain; the mtDNA deletions, however, were found to be decreased with respect to age-matched controls, shedding doubt on the significance of the deletions, at least in concern with the disease [210].

A severe compromission of complex I has been found in the substantia nigra of subjects with Parkinson's disease [211], and also, though less evident,

in muscle and in platelets and lymphocytes of diseased individuals [207,212]. The lowered levels of reduced glutathione in the substantia nigra of asymptomatic Parkinson's carriers with Lewy bodies [213] militates for an early involvement of oxidative stress in the pathogenesis of the disease [83]. The involvement of mtDNA deletions or mutations is not proven with certainty [214,215]. It was suggested that certain point mutations occurring as polymorphisms in mtDNA may serve as risk factors for neurodegenerative diseases [216]; the concomitant lesions due to ageing and to environmental stress would gradually impair mitochondrial function leading to excitotoxic cell death and progressive atrophy of the interested area. The complex I defect present in platelets from Parkinson patients was transferred to fusion cybrids, indicating a possible mtDNA defect [217]. The fact that some brain areas seem to be more susceptible to mtDNA damage in comparison with others may be related to the specific localization of neurodegenerative diseases [83]. The picture is emerging that most degenerative diseases originate from a concomitance of risk factors enhancing the danger of oxidative stress and energy failure in specific tissues.

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EXHIBIT 3

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Increased Oxidative Damage Is Correlated to Altered Mitochondrial Function in Heterozygous Manganese Superoxide Dismutase Knockout Mice*

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This study characterizes mitochondria isolated from livers of $Sod2^{-/+}$ and $Sod2^{+/+}$ mice. A 50% decrease in manganese superoxide dismutase (MnSOD) activity was observed in mitochondria isolated from $Sod2^{-/+}$ mice compared with $Sod2^{+/+}$ mice, with no change in the activities of either glutathione peroxidase or copper/zinc superoxide dismutase. However, the level of total glutathione was 30% less in liver mitochondria of the $Sod2^{-/+}$ mice. The reduction in MnSOD activity in $Sod2^{-/+}$ mice was correlated to an increase in oxidative damage to mitochondria: decreased activities of the Fe-S proteins (aconitase and NADH oxidoreductase), increased carbonyl groups in proteins, and increased levels of 8-hydroxydeoxyguanosine in mitochondrial DNA. In contrast, there were no significant changes in oxidative damage in the cytosolic proteins or nuclear DNA. The increase in oxidative damage in mitochondria was correlated to altered mitochondrial function. A significant decrease in the respiratory control ratio was observed in mitochondria isolated from $Sod2^{-/+}$ mice compared with $Sod2^{+/+}$ mice for substrates metabolized by complexes I, II, and III. In addition, mitochondria isolated from $Sod2^{-/+}$ mice showed an increased rate of induction of the permeability transition. Therefore, this study provides direct evidence correlating reduced MnSOD activity *in vivo* to increased oxidative damage in mitochondria and alterations in mitochondrial function.

Under normal physiological conditions, metabolism of oxygen by aerobic organisms generates a wide variety of potentially deleterious reactive oxygen species. These reactive oxygen species initiate a large number of oxidative reactions in cellular systems that lead to the oxidation of macromolecules, *e.g.* DNA, proteins and lipids (1). Over the past decade, numerous investigators have argued that oxidative damage may contribute to a variety of chronic diseases (including emphysema, cardiovascular disease, cancer, and neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis) as well as aging (2). Although it is attractive to speculate that the accumulation of oxidative damage may be involved in the decline of physiological function and increased disease pathogenesis, there is no direct evidence for this concept.

Mitochondria are extremely susceptible to oxidative damage because 2–4% of the oxygen consumed by mitochondria is converted to superoxide anions by the electron transport chain (3) and because mitochondria have limited protection from oxidative stress (4). Therefore, mitochondria would be predicted to be one of the cellular components especially vulnerable to oxidative damage. Over the past 20 years, investigators have studied the effect of oxidative damage on mitochondrial function by subjecting isolated mitochondria to oxidative stress *in vitro*. Many functional properties of mitochondria are substantially impaired after exposure *in vitro* to a variety of reactive oxygen species. These include the inhibition of the complexes in the respiratory chain, especially complex I and ATP synthetase (5), decreased adenine nucleotide content, inhibition of the adenine nucleotide translocase (6), increased lipid peroxidation (7), and mitochondrial swelling (8). However, it is not clear that *in vitro* studies with isolated mitochondria are an accurate indication of what occurs *in vivo*. In other words, does the accumulation of oxidative damage under normal physiological conditions *in vivo* lead to mitochondrial dysfunction?

Transgenic or mutant animals with alterations in the antioxidant defense system provide investigators with a model system that can be used to test the effect of oxidative damage on cellular function *in vivo*. For example, two MnSOD[1] knockout mouse models have been produced by the disruption of different sections of the *Sod2* gene (9, 10). In both knockout models, the homozygous ($Sod2^{-/-}$) mutants exhibited no detectable MnSOD activity in any of the tissues studied. The $Sod2^{-/-}$ phenotype was lethal in both knockout models. The $Sod2^{-/-}$ mutants (*Sod2*<tm1>Cje) produced by Li *et al.* (9) were smaller and paler and exhibited a hypotonic and hypothermic state compared with wild type mice and the heterozygous mice ($Sod2^{-/+}$). In addition to their overall gross appearance, the homozygous null mutants appeared to fatigue more rapidly after any type of exertion; however, their behavior was otherwise normal. After 4–5 days, 37% of the $Sod2^{-/-}$ mice died, with nearly all the animals dead by day 10. At death, the $Sod2^{-/-}$ mice had enlarged hearts with a dilated left ventricular cavity and reduced left ventricular wall thickness, which

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[1] The abbreviations used are: MnSOD, manganese superoxide dismutase; mtDNA, mitochondrial DNA; GPX, glutathione peroxidase; CoQ, coenzyme Q_1; RCR, respiratory control ratio; 8-OHdG, 8-hydroxydeoxyguanosine.

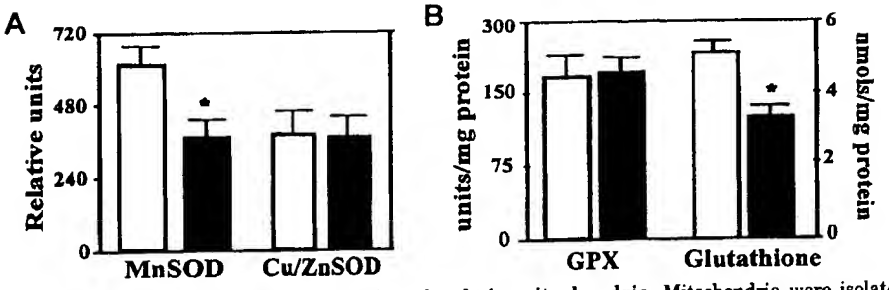


FIG. 1. **Antioxidant enzyme activities and total glutathione levels in mitochondria.** Mitochondria were isolated from the livers of $Sod2^{+/+}$ (*open bars*) and $Sod2^{-/+}$ (*shaded bars*) mice. *A* shows the activities of MnSOD and copper/zinc superoxide dismutase. *B* shows activities of GPX and the levels of total glutathione in the isolated mitochondrial preparations. All values reported are the mean ± S.E. of five to eight animals. *, $p < 0.05$ by paired Student's *t* test.

from $Sod2^{-/+}$ compared with $Sod2^{+/+}$ mice. In contrast, the activity of GPX remained unchanged (Fig. 1) and, therefore, did not compensate for the reduced activity of MnSOD in the liver mitochondria of the $Sod2^{-/+}$ mice. We also measured the CuZnSOD and catalase activities in these mitochondrial preparations. CuZnSOD has been reported to be localized not only in the cytosol, but also in the intermembrane space of the mitochondria (25). The activity of CuZnSOD was the same for the $Sod2^{-/+}$ and $Sod2^{+/+}$ mice (Fig. 1). These data are in agreement with the previous report by Li *et al.* (9), in which no difference in CuZnSOD activity was observed in whole liver homogenates in these mice. There was no detectable catalase activity in the mitochondrial extracts from either the $Sod2^{+/+}$ or $Sod2^{-/+}$ mice. This was not unexpected because catalase has been reported to be localized in the cytosol, specifically the perixosomes (26).

We also measured total glutathione levels in isolated mitochondria because glutathione can act directly as an antioxidant or as a substrate for GPX. Only 10–15% of the total cellular glutathione is found inside the mitochondrial matrix (27); therefore, it was only possible to measure total glutathione levels in the mitochondrial extracts. The data in Fig. 1 show that total glutathione levels were decreased approximately 30% in mitochondria from the $Sod2^{-/+}$ mice compared with mitochondria from $Sod2^{+/+}$ mice. This decrease could be of significance because the mitochondrial glutathione pool appears to be extremely important physiologically in protecting cells from oxidative stress. For example, Garcia-Ruiz *et al.* (28) recently showed that a decrease in the mitochondrial pool of glutathione (with the cytosolic glutathione pool intact) rendered cells more vulnerable to the endogenous oxidative stress induced by antimycin A compared with the situation when only the cytosolic glutathione pool was decreased.

Oxidative Damage—Because MnSOD is a key component of the antioxidant defense system, we determined if the reduction in MnSOD activity in the mitochondria of the $Sod2^{-/+}$ mice was correlated to a change in oxidative damage. We measured the activity of aconitase because it has been reported to be a sensitive measure of tissue/cellular levels of superoxide anions (29). Aconitase is an iron-sulfur protein that is inactivated by superoxide anions (29), which oxidizes the [4Fe-4S] cluster and leads to a loss of an iron ion (30). Aconitase inactivated by superoxide anions can be reactivated by the addition of a reducing agent and iron (15). Fig. 2 shows that the mitochondrial aconitase activity is reduced 30% in mitochondrial extracts from the livers of $Sod2^{-/+}$ mice compared with $Sod2^{+/+}$ mice. However, the Western blot in Fig. 2 shows that levels of aconitase protein in the mitochondrial extracts from the $Sod2^{-/+}$ and $Sod2^{+/+}$ were similar; therefore, the decrease in aconitase activity was not due to reduced levels of the protein. The data in Fig. 2 also show that reactivation of aconitase by the addition of iron and dithiothreitol resulted in an increase in the mitochondrial aconitase activity in the $Sod2^{-/+}$ mice to a level equal to that measured in the $Sod2^{+/+}$ mice. Therefore, the decrease in aconitase activity in the liver mitochondria of the $Sod2^{-/+}$ mice appears to be the result of inactivation by superoxide anions. In other words, superoxide anion levels in the mitochondria of the $Sod2^{-/+}$ mice appear to be higher than the levels in the mitochondria of the $Sod2^{+/+}$ mice. Previously, Li *et al.* (9) reported no difference in the total aconitase activity in the liver homogenates from $Sod2^{-/+}$ and $Sod2^{+/+}$ mice; however, it is possible that they failed to detect changes in aconitase activity because they measured total rather than mitochondrial aconitase activity. There are approximately equal activities of the mitochondrial and cytosolic aconitase in liver (31).

We also measured the activity of NADH oxidoreductase (complex I) in mitochondrial extracts because the [Fe-S] clusters in the complexes are sensitive to oxidative inactivation (21, 32). NADH oxidoreductase activity was measured by two assays that employ different terminal electron acceptors: ferricyanide and coenzyme Q (Table I). Coenzyme Q, a ubiquinone analog, accepts electrons from the [Fe-S] clusters of complex I; these clusters are sensitive to oxidative inactivation. The activity of NADH oxidoreductase using coenzyme Q as the substrate was significantly lower (30%) in the mitochondria isolated from the livers of the $Sod2^{-/+}$ mice compared with $Sod2^{+/+}$ mice (Table I). To determine if the loss in activity was due to inactivation of the [Fe-S] clusters, we also measured the NADH oxidoreductase activity using ferricyanide as the substrate. Ferricyanide is an artificial electron acceptor that accepts electrons directly from the reduced flavin mononucleotide and by-passes the [Fe-S] clusters of complex I (33). Using ferricyanide as the terminal electron acceptor, no difference in the activities of NADH oxidoreductase was observed for mitochondria isolated from the livers of $Sod2^{-/+}$ and $Sod2^{+/+}$ mice (Table I). Therefore, the decrease in NADH oxidoreductase activity appears to be due to oxidation of the [Fe-S] clusters.

We also measured the activities of fumarase in the mitochondrial extracts as a control because fumarase is not iron-dependent (34) and it is insensitive to oxidative inactivation (35). The data in Table I show that fumarase activity was the same in the mitochondrial preparations from the $Sod2^{-/+}$ and $Sod2^{+/+}$ mice even though aconitase and NADH oxidoreductase activities were significantly reduced in the $Sod2^{-/+}$ mice.

We also measured the activities of aconitase and glutamine synthetase in the cytosolic extracts isolated from the livers of the $Sod2^{-/+}$ and $Sod2^{+/+}$ mice. The mitochondrial and cytosolic aconitase are encoded by two different genes; however, the [4Fe-4S] structure is conserved, and cytosolic aconitase is sensitive to oxidative inactivation by superoxide anions (36). Glutamine synthetase is a cytosolic enzyme that has been shown to be sensitive to oxidative inactivation (37, 38) because a single histidine residue has been oxidized producing a carbonyl group

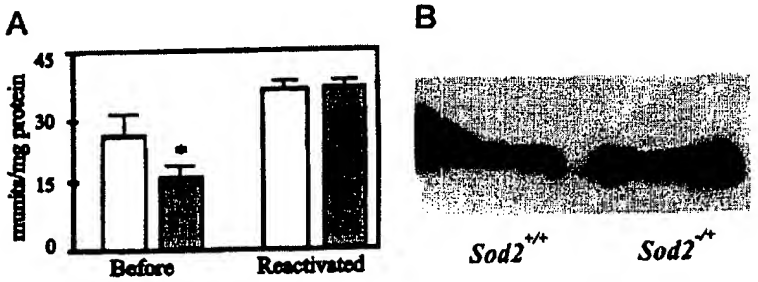


FIG. 2. **Aconitase activities and protein levels in isolated mitochondria.** *A* shows aconitase activities in liver mitochondria from $Sod2^{+/+}$ (*open bars*) and $Sod2^{-/+}$ (*shaded bars*) mice before and after reactivation by the addition of dithiothreitol and iron as described under "Materials and Methods." The values represent the mean ± S.E. of eight animals. *, $p < 0.05$ by paired Student's *t* test. *B* shows the autoradiograph of a Western blot using a polyclonal antibody developed against aconitase. Mitochondrial proteins were separated electrophoretically under reducing conditions by SDS-polyacrylamide gel electrophoresis, and the blot was developed using a secondary antibody coupled to the horseradish peroxidase ECL system (Amersham, Les Ulis, France) as described by Heydari *et al.* (48).

TABLE I
The activities of mitochondrial and cytosolic enzymes in the liver of $Sod2^{+/+}$ and $Sod2^{-/+}$ mice

All values represent the mean ± S.E. of six to nine animals. *, $p < 0.01$ by paired Student's *t* test.

	$Sod2^{+/+}$	$Sod2^{-/+}$
Mitochondrial enzymes		
NADH oxidoreductase[a]		
NADH:coenzyme Q	309.6 ± 25.7	216.1 ± 9.8*
NADH:ferricyanide	626.9 ± 118.7	626.9 ± 121.7
Fumarase[a]	161.5 ± 13.8	184.2 ± 20.2
Cytosolic enzymes		
Aconitase[a]	38.3 ± 2.3	38.8 ± 2.0
Glutamine synthetase[b]	52.5 ± 5.4	54.2 ± 7.1

[a] Milliunits/mg of mitochondrial protein.
[b] Units/mg of mitochondrial protein.

(39). The data in Table I show no difference in the activity of either enzyme in the cytosol of the $Sod2^{+/+}$ or $Sod2^{-/+}$ mice.

To further characterize the level of protein oxidation in the mitochondrial and cytosolic fractions of the liver from $Sod2^{+/+}$ and $Sod2^{-/+}$ mice, we measured the levels of carbonyl groups in specific proteins by Western blot analysis using an antibody that detects 2,4-dinitrophyenyl adducts (40). Carbonyl groups (aldehydes and ketones) are formed in the amino side chains of proteins by metal catalyzed oxidation and are standard markers of protein oxidation (41). The Western blot in Fig. 3 shows that more carbonyl groups were present in mitochondrial extracts from the $Sod2^{-/+}$ mice compared with extracts from the $Sod2^{+/+}$ mice. This difference was not due to differences in protein levels as shown in the Coomassie Blue stain in Fig. 3*B*. In contrast to mitochondrial proteins, cytosolic protein extracts contain similar levels of carbonyl groups in the $Sod2^{-/+}$ and $Sod2^{+/+}$ mice. To ensure that the antibody specifically detected carbonyl groups, a derivatization control was included in which no 2,4-dinitrophenyl hydrazine was added to the extracts. This resulted in a loss of binding of the antibody in both the cytosolic and mitochondrial extracts.

We also compared levels of oxidative damage in the livers of the $Sod2^{-/+}$ and $Sod2^{+/+}$ mice by measuring the levels of 8-hydroxydeoxyguanosine (8-OHdG) in mtDNA and nuclear DNA. The data in Fig. 4 show that the level of 8-OHdG in mtDNA from the livers of the $Sod2^{-/+}$ mice was significantly higher (30%) than that found in mtDNA from the $Sod2^{+/+}$ mice. In contrast, the levels of 8-OHdG in nuclear DNA were similar in the $Sod2^{-/+}$ and $Sod2^{+/+}$ mice.

Mitochondrial Function—Because mitochondria from the $Sod2^{-/+}$ mice showed higher levels of oxidative damage, we compared the function of mitochondria isolated from the livers of $Sod2^{-/+}$ and $Sod2^{+/+}$ mice. The rates of state 3 and state 4 respiration and the RCR were measured because they are

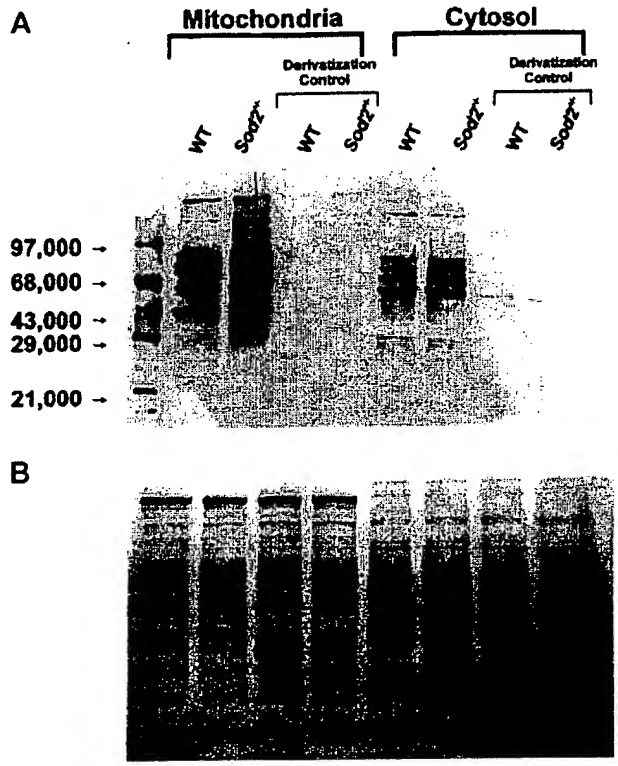


FIG. 3. **Oxidatively modified proteins containing carbonyl groups in mitochondria and cytosol.** Mitochondrial and cytosolic extracts from livers of $Sod2^{+/+}$ (*WT*) and $Sod2^{-/+}$ mice were analyzed by SDS-polyacrylamide gel electrophoresis using the Oncor Oxyblot kit (Oncor, Gaithersburg, MD) as described by Keller *et al.* (40). *A* shows the autoradiograph of a Western blot using a polyclonal antibody developed against carbonyl groups. *B* shows the Coomassie Blue stain of the Western blot in *A*. To ensure that the antibody specifically detected carbonyl groups, a derivatization control was included in which no 2,4-dinitrophenyl hydrazine was added to the extracts.

measurements of the efficiency of the movement of electrons along the electron transport chain and the coupling of this movement to the production of ATP by oxidative phosphorylation (24). Oxygen consumption of isolated mitochondria was measured using substrates that are metabolized through different complexes in the electron transport chain, and Table II shows the rates of state 3 and state 4 respiration with the three substrates. The RCR was significantly lower for mitochondria isolated from $Sod2^{-/+}$ mice for all three substrates. The decrease in RCR was greatest (29 and 33%) for the substrates glutamate/malate and duroquinol, which are metabolized

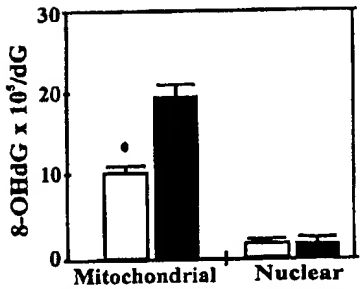


FIG. 4. **DNA oxidation of mtDNA and nuclear DNA.** Nuclear and mitochondrial DNA were isolated from livers of $Sod2^{+/+}$ (*open bars*) and $Sod2^{-/+}$ (*shaded bars*) mice. The concentrations of 8-OHdG and deoxyguanosine (*dG*) in the DNA hydrolysates were determined using high performance liquid chromatography and quantified by electrochemical detection as described by Floyd *et al.* (49). A CoulChem® electrochemical detection system (ESA model 5200, ESA, Inc., Chelmsford, MA) was used with a reverse phase, isocratic system as described by Beal *et al.* (50). The ratios of 8-OHdG/dG in liver nuclear and mitochondrial DNA are shown. Each value represents the mean ± S.E. of six different experiments, in which mitochondria were pooled from two animals in each experiment. *, $p < 0.05$ by paired Student's *t* test.

TABLE II
Oxygen utilization in mitochondria isolated from the livers of $Sod2^{+/+}$ and $Sod2^{-/+}$ mice

Each value represents the mean ± S.E. of six different experiments, in which mitochondria were pooled from two animals per experiment. *, $P < 0.05$ by paired Student's *t* test. **, $P < 0.01$ by paired Student's *t* test.

Substrate	State 3[a]	State 4[a]	RCR
Glutamate/malate			
$Sod2^{+/+}$	30.7 ± 3.0	6.5 ± 0.8	5.1 ± 0.5
$Sod2^{-/+}$	22.7 ± 3.9	6.8 ± 0.9	3.4 ± 0.5**
Succinate			
$Sod2^{+/+}$	39.2 ± 5.7	14.8 ± 2.0	2.6 ± 0.1
$Sod2^{-/+}$	29.9 ± 3.9	14.2 ± 1.9	2.2 ± 0.1**
Duroquinol			
$Sod2^{+/+}$	57.0 ± 10.2	18.7 ± 3.0	3.1 ± 0.3
$Sod2^{-/+}$	32.2 ± 8.3*	16.5 ± 5.5	2.2 ± 0.2*

[a] nmol O_2/min/mg protein.

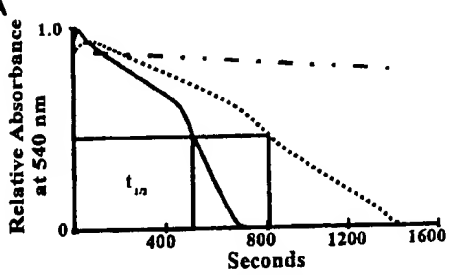
through complexes I and III, respectively. This is not surprising because these two complexes have been reported to be more sensitive to oxidative damage for the following reasons: (*a*) they contain [Fe-S] clusters that are sensitive to oxidative stress (42), (*b*) they are the major sites for the production of reactive oxygen species (43), and (*c*) cardiolipin, which is an essential for the biological activities of these complexes, is sensitive to peroxidation by reactive oxygen species (44). The respiration data with glutamate/malate are also consistent with our data in Table II, which showed that the activity of NADH oxidoreductase with coenzyme Q as a substrate was reduced approximately 30%.

The decrease in the RCR appears to be due to a decrease in state 3 respiration. The data in Table II show a significant decrease (44%) in state 3 respiration with duroquinol as a substrate in the $Sod2^{-/+}$ mice compared with the $Sod2^{+/+}$ mice. The substrates succinate and glutamate/malate did not show a statistical significant decrease in state 3 respiration in the $Sod2^{+/+}$ mice; however, the decrease in state 3 respiration with glutamate/malate as a substrate approached statistical significance with a *P* value of 0.06. The rates of state 4 respiration are essentially identical in the mitochondria isolated from $Sod2^{-/+}$ and $Sod2^{+/+}$ mice, which suggests there was no disruption in membrane integrity in the inner mitochondrial membrane of the $Sod2^{-/+}$ mice (45). This observation was further substantiated when we measured the membrane potential of mitochondria isolated from $Sod2^{-/+}$ and $Sod2^{+/+}$ mice using safranine O as described by Akerman and Wikstrom (46). The membrane potentials for mitochondria isolated from the livers of $Sod2^{-/+}$ and $Sod2^{+/+}$ mice were −173.74 ± 15.61 mV and −167.30 ± 23.93 mV, respectively (data are expressed as the mean ± S.E. from six experiments pooling mitochondria from two livers for each experiment).

Mitochondria isolated from the $Sod2^{-/+}$ mice also showed altered function with respect to the induction of the permeability transition. The permeability transition occurs through a proteinaceous pore, whose opening is induced by calcium and oxidative stress, such as *t*-butylhydroperoxide (47). The induction of the mitochondria permeability transition pore is characterized by a sudden increase in the permeability of the mitochondrial inner membrane to small ions and molecules (calcium and glutathione) that can lead to a complete collapse of the membrane potential and swelling of the matrix. Fig. 5*A* shows that mitochondria isolated from the livers of $Sod2^{-/+}$ mice undergo more rapid swelling in the presence of calcium and *t*-butylhydroperoxide based upon the decrease in relative absorbance. To ensure that the increased rate of mitochondrial swelling was due to the induction of the permeability transition, cyclosporin A, an inhibitor of the permeability transition pore (42), was added to the reactions. As illustrated in Fig. 5, the addition of cyclosporin A inhibited the decrease in absorbance, indicating that the changes in absorbance were due to the induction of the permeability transition pore. Fig. 5 shows that the rate of induction (calculated as $t_{1/2}$ in seconds) of the permeability transition by calcium was higher for mitochondria isolated from the livers of $Sod2^{-/+}$ mice. However, this increase was not statistically significant. Addition of both *t*-butylhydroperoxide and calcium increased the rate of induction of the permeability transition for mitochondria isolated from both the $Sod2^{+/+}$ and $Sod2^{-/+}$ mice. The data in Fig. 5 show that the rate of induction of the permeability transition was significantly higher (38%) for mitochondria isolated from the $Sod2^{-/+}$ mice compared with $Sod2^{+/+}$ mice in the presence of calcium and *t*-butylhydroperoxide. The more rapid induction of the mitochondrial transition pore in the $Sod2^{-/+}$ mice potentially could be serious physiologically because it could lead to the loss of mitochondrial membrane integrity. Once mitochondria can no longer maintain a proton-motive force, they are unable to generate ATP.

In summary, our data provides more direct and detailed evidence that the decrease in MnSOD activity in liver mitochondria of $Sod2^{-/+}$ mice is physiologically important. We found that superoxide anion levels were increased in the mitochondria from $Sod2^{-/+}$ mice as measured by a loss of aconitase activity. The increased levels of superoxide anions would be predicted to lead to increased oxidative damage, and we have presented several lines of evidence showing that mitochondria from $Sod2^{-/+}$ mice experience greater oxidative stress/damage. Glutathione levels are reduced, NADH oxidoreductase activity is reduced, carbonyl groups in mitochondrial proteins are increased, 8-OHdG levels in mtDNA are increased, and the induction of the permeability transition is increased. Interestingly, we were unable to detect any evidence for changes in oxidative stress/damage in the cytosol and nuclei of liver from the $Sod2^{-/+}$ mice. Oxidation of cytosolic proteins (glutamine synthetase activity and carbonyl groups) and nuclear DNA (8-OHdG) are similar in the $Sod2^{-/+}$ and $Sod2^{+/+}$ mice. Thus, it appears that the physiological impact of the mutation in the $Sod2^{-/+}$ mice is limited to the mitochondria, which is further evidence for MnSOD playing a critical role in the first line of defense against superoxide anions that are produced during normal aerobic respiration in mitochondria. One of the major observations from our study was that the function of mitochondria from the $Sod2^{-/+}$ mice was compromised. Mitochondria

A



B

	Inducers	
	Calcium	Calcium and *t*-Butylhydroperoxide
Sod2$^{+/+}$	1370 ± 125	800 ± 65
Sod2$^{-/+}$	1126 ± 151	495 ± 57*

FIG. 5. **Induction of the mitochondrial permeability transition.** The induction of the permeability transition was determined by following mitochondrial swelling as measured by a decrease in absorbance at 540 nm (51). *A* is a representative tracing of the induction of the permeability transition with 100 μM calcium chloride and 75 μM *t*-butylhydroperoxide for mitochondria isolated from the livers of *Sod2*$^{-/+}$ (——) and *Sod2*$^{+/+}$ (--) mice. Cyclosporin A (·--·) at a concentration of 500 nM was added as a control to inhibit the induction of the permeability transition by calcium and *t*-butylhydroperoxide. The time in seconds at which one-half of the absorbance was lost ($t_{1/2}$) was calculated and used as a measure of the permeability transition. The table in *B* shows the $t_{1/2}$ in seconds for the induction of the permeability transition for mitochondria isolated from *Sod2*$^{+/+}$ and *Sod2*$^{-/+}$ mice. Each value represents the mean ± S.E. of six separate experiments in which mitochondria were pooled from two animals for each experiment. *, $p < 0.05$ by paired Student's *t* test.

isolated from the livers of *Sod2*$^{-/+}$ mice showed reduced respiratory control ratios with substrates that are metabolized by complexes I, II, and III. In other words, it appears that the coupling of oxygen consumption to ATP production is less efficient for liver mitochondria from *Sod2*$^{-/+}$ mice. Thus, we have strong *in vivo* evidence that increased oxidative damage leads to reduced mitochondrial function. Our data *in vivo* are consistent with the data that has been obtained *in vitro* over the past 20 years showing that mitochondrial function is substantially impaired when mitochondria are exposed to oxidative stress (5).

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EXHIBIT 4

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Age-associated Change in Mitochondrial DNA Damage

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There is an age-associated decline in the mitochondrial function of the Wistar rat heart. Previous reports from this lab have shown a decrease in mitochondrial cytochrome c oxidase (COX) activity associated with a reduction in COX gene and protein expression and a similar decrease in the rate of mitochondrial protein synthesis. Damage to mitochondrial DNA may contribute to this decline.

Using the HPLC–Coularray system (ESA, USA), we measured levels of nuclear and mitochondrial 8-oxo-2′-deoxyguanosine (8-oxodG) from 6-month (young) and 23-month-old (senescent) rat liver DNA. We measured the sensitivity of the technique by damaging calf thymus DNA with photoactivated methylene blue for 30 s up to 2 h. The levels of damage were linear over the entire time course including the shorter times which showed levels comparable to those expected in liver. For the liver data, 8-oxodG was reported as a fraction of 2-deoxyguanosine (2-dG). There was no change in the levels of 8-oxodG levels in the nuclear DNA from 6 to 23-months of age. However, the levels of 8-oxodG increased 2.5-fold in the mitochondrial DNA with age. At 6 months, the level of 8-oxodG in mtDNA was 5-fold higher than nuclear and increased to approximately 12-fold higher by 23 months of age. These findings agree with other reports showing an age-associated increase in levels of mtDNA damage; however, the degree to which it increases is smaller. Such damage to the mitochondrial DNA may contribute to the age-associated decline in mitochondrial function.

Keywords: Mitochondrial DNA, oxidative damage, DNA repair, mitochondria, aging, liver

THE MITOCHONDRIAL GENOME AND CYTOCHROME *c* OXIDASE

Mitochondria contain their own circular genome that encodes 2 rRNAs, 22 tRNAs, and 13 polypeptides. These polypeptides are subunits of the respiratory chain complexes located in the mitochondrial inner membrane.[1] With its own genome, the mitochondrion is capable of translation, transcription, and DNA synthesis. However, all of these processes are dependent on cytosolic components of nuclear origin such as heat-shock proteins, initiation factors, elongation factors,

*Corresponding author.

transcription factors, polymerases and DNA repair enzymes[2,3] (Figure 1).

Cytochrome oxidase (COX), the terminal activity of the respiratory chain and essential for proper mitochondrial function, is composed of 13 subunits of which the three catalytic subunits are encoded by the mitochondrial genome. Therefore, a decline in mitochondrial translation or transcription of DNA with age could result in reduced in COX activity.

In addition, damage to mitochondrial DNA (mtDNA) could play a role in the decrease in COX function. One of the major theories of aging states that oxidative damage to mitochondrial macromolecules, including mtDNA, decreases mitochondrial function thereby lowering the cell's energy supply leading to cell death.[4] DNA damage could reach deleterious levels if there was a reduction in repair activity or if DNA damage levels simply overwhelmed the repair capacity of the mitochondria. Such a loss of mtDNA repair and/or increase in mtDNA damage could adversely affect COX function.

Previous reports have shown an age-associated decline in COX activity.[5,6] After confirming that there was a decrease in COX activity with age, we examined other mitochondrial processes to determine if some aspect of mitochondrial gene expression could account for this change in function. Using isolated mitochondria, we investigated both mitochondrial protein synthesis and transcription to determine if either or both played a role in the decline of COX activity.

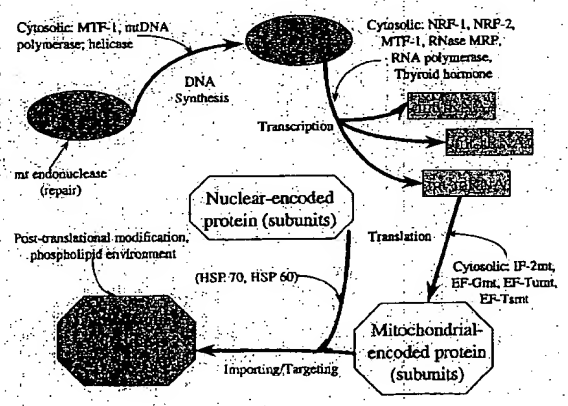

FIGURE 1 *The process of mitochondrial biogenesis.* This flow chart illustrates the complexity of the interaction between the nuclear and mitochondrial genomes required for mitochondrial biogenesis and function. The "backbone", shown with the thick black lines, represents the events that take place within the mitochondrion. Those activities and proteins of non-mitochondrial origin are labeled in blue and are required at numerous points in the pathway as indicated by the small arrows. They include transcription, initiation, and elongation factors as well as the heat shock proteins needed for mitochondrial biogenesis. In addition, numerous nuclear encoded subunits, such as those of cytochrome oxidase and the ATP synthase, and proteins are required for proper mitochondrial function. (See Color plate I at the end of this issue.)

CHANGES IN MITOCHONDRIAL PROTEIN SYNTHESIS AND GENE EXPRESSION WITH AGE

To verify that there was an age-associated decrease in COX function in our model, we isolated rat heart mitochondria and compared cytochrome oxidase activities in young, 6–7-month old rats and old, 23–24-month old rats. In agreement with previous reports, there was a 30% decrease in COX activity with age.[5,6] Citrate synthase failed to show an age-associated change in activity suggesting that the decline in COX activity was the result of the reduced specific activity of the enzyme and did not reflect differential contamination of the preparation. The decrease in COX activity was associated with a decrease in the protein levels of the COX subunits and a lower rate of mitochondrial protein synthesis with age.[7] There was a similar decrease in the mRNA expression of the COX subunits and a reduction in the rate of mitochondrial transcription.[7] Combined, these decreases in mitochondrial function could account for the decrease in COX activity with age. However, we wanted to determine if damage to mtDNA could also play a role in this decline.

OXIDATIVE DAMAGE TO OTHER CELLULAR COMPONENTS

The free radical theory of aging has become part of the fabric of the scientific community.[8] Because they are one of the major sources of reactive oxygen species in the cell, mitochondria are believed to play a role in the aging process.[4] It has been shown that other cellular macromolecules such as proteins, phospholipids, and nucleic acids suffer oxidative damage.[9–11] We examined damage to mitochondria by measuring lipid peroxidation in isolated mitochondria using the thiobarbituric acid reactive species (TBARS) assay.[12] This spectrophotometric assay measures levels of malondialdehyde (MDA), a secondary product of lipid peroxidation.

The results show no change in mitochondrial TBARS levels indicating no increase in lipid peroxidation in the aging rat heart. However, the sensitivity of this spectrophotometric assay has been brought into question. The problem is that other aldehydes may react with TBA giving spurious results. It is possible that the best method for measuring lipid peroxidation may be the separation of other aldehydes in the sample by HPLC followed by a spectrophotometric analysis or electrochemical analysis of the lipid peroxidation product 4-hydroxynonenal.[13] The increased sensitivity of these methods may show a difference in lipid peroxidation levels.

CHANGES IN MITOCHONDRIAL DNA PROCESSING WITH AGE

Our lab recently characterized a protein called mitochondrial oxidative damage-specific endonuclease (mtODE).[14] We have purified and defined an endonuclease with 8-oxoguanine specificity that was located within the mitochondria. We thought mtODE could provide some insight into age-associated changes in mtDNA processing and/or mtDNA repair. Due to reports showing an increase in mtDNA damage with age, we expected to find a decrease in mtODE activity.[15–17] Using a radiolabeled oligo containing 8-oxoguanine, we tested mitochondrial preparations from young and old rat hearts for mtODE activity.[18]

We found there to be a 40% *increase* in mtODE activity with age.[18] This lead us to speculate that an increase in DNA damage somehow led to an increase in the expression or activity of DNA processing enzymes. To test this hypothesis, we began isolating DNA from rat heart to confirm that there were higher levels of oxidative DNA damage with age. Efforts to determine the levels of oxidative damage to mtDNA were hampered by the inability to isolate appropriate amounts of mtDNA for HPLC–EC analysis from two rat hearts. For reasons not yet understood, most of the heart mtDNA failed to precipitate out of the ethanol. We are currently trying to modify and optimize the method using the smallest number of rats possible.

To test our hypothesis, we began using isolated rat liver mitochondria and began the process of characterizing liver mtODE activity hoping it would have the same increase in activity as that seen in heart. As was the case with heart, liver mtODE activity increased 40% with age.[18] So we felt confident that we could test our theory and began the isolation of both nuclear and mitochondrial DNA from rat liver.

PREPARATION AND ANALYSIS OF LIVER DNA

Liver mitochondria were isolated by differential centrifugation for the preparation of mtDNA, and the pellet of the first low speed spin was used for the preparation of nuclear DNA. The DNA was isolated using the standard RNAase A, proteinase K, and phenol chloroform procedure with some modifications which generated enough DNA for analysis (200–500 μg).[19] Nucleosides were prepared enzymatically

from 100 µg of DNA using nuclease P1 and alkaline phosphatase then filtered through a 0.22-micron filter and a 30 kD cutoff spin column.[20]

To determine the levels of DNA damage, we monitored levels of 8-oxodG, the oxidative product of 2-dG, in the nuclear and mitochondrial DNA samples.[21] This electrochemically active DNA adduct was measured using an ESA four-channel coularray with two channels set at low potentials for the detection of 8-oxodG and two channels set at higher potentials for the detection of 2-dG. The midpoint potentials of the two compounds, 8-oxodG and 2-dG, differed by 400 mV simplifying identification (Figure 2). The levels of 2-dG were too high to be measured in the same run, so the samples were diluted 1:100 in mobile phase then analyzed in a separate run for 2-dG. All samples were analyzed twice.

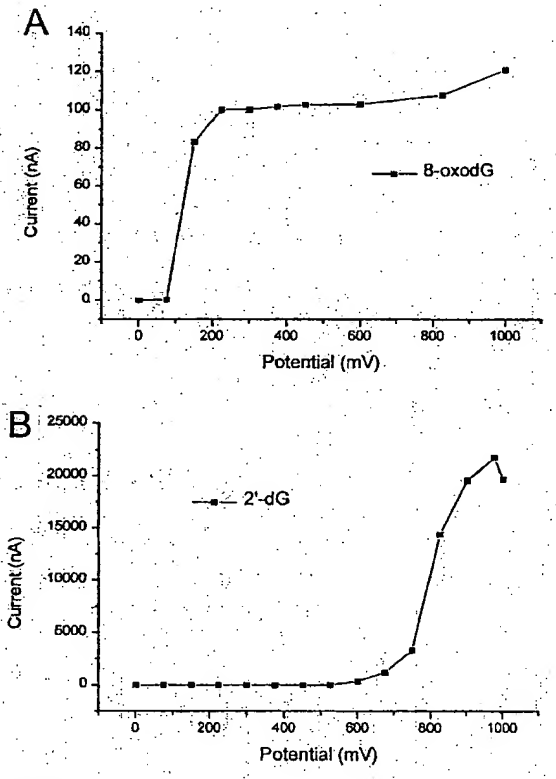

FIGURE 2 *Hydrodynamic voltammograms (HDVs) of 8-oxodG and 2-dG.* The HDV is a current–voltage curve that indicates the specific voltage at which a compound is oxidized and produces a current. Using standards for 8-oxodG and 2-dG, we found the optimum potential for 2-dG to be around 950 mV (panel A) and for 8-oxodG it was around 300 mV (panel B). This allows accurate identification and separation of the two DNA adducts.

The mobile phase was composed of 100 mM sodium acetate, pH 5.15 and 5% methanol. The nucleosides were separated isocratically using a C-8 column at a flow rate of 1 ml/min for 30 min to avoid spillover to the successive run. Nucleoside standards were run first and had approximately a 70:30 ratio over the two channels. The concentrations of the standards were determined spectrophotometrically. The peaks were identified in the samples according to the retention time and ratio accuracy. For our biological samples, the 8-oxodG was expressed per 10^5 2-dG.

To illustrate the sensitivity and accuracy of the system, calf thymus DNA was damaged with photoactivated methylene blue for up to 2 h then analyzed for 8-oxodG content.[22,23] Damage increased in a linear fashion over the entire 2-h time course, and we were able to detect low levels of damage with only a 30 s of light exposure. More importantly, we were able to confidently detect levels in the fmol range needed for biological samples (Figure 3).

To confirm that our peak of interest was indeed 8-oxodG, we damaged rat liver DNA with methylene blue and light, then split the sample in two and treated one-half with Fpg to remove 8-oxodG.[24] We analyzed our peak of interest and found that Fpg reduced the signal by 90% thus confirming our peak was 8-oxodG.[25] We then analyzed our liver DNA.

AGE-ASSOCIATED INCREASE IN mtDNA DAMAGE

Our results show an approximate 2.5-fold increase in mitochondrial 8-oxodG and no change in nuclear levels with age. At 6 months of age,

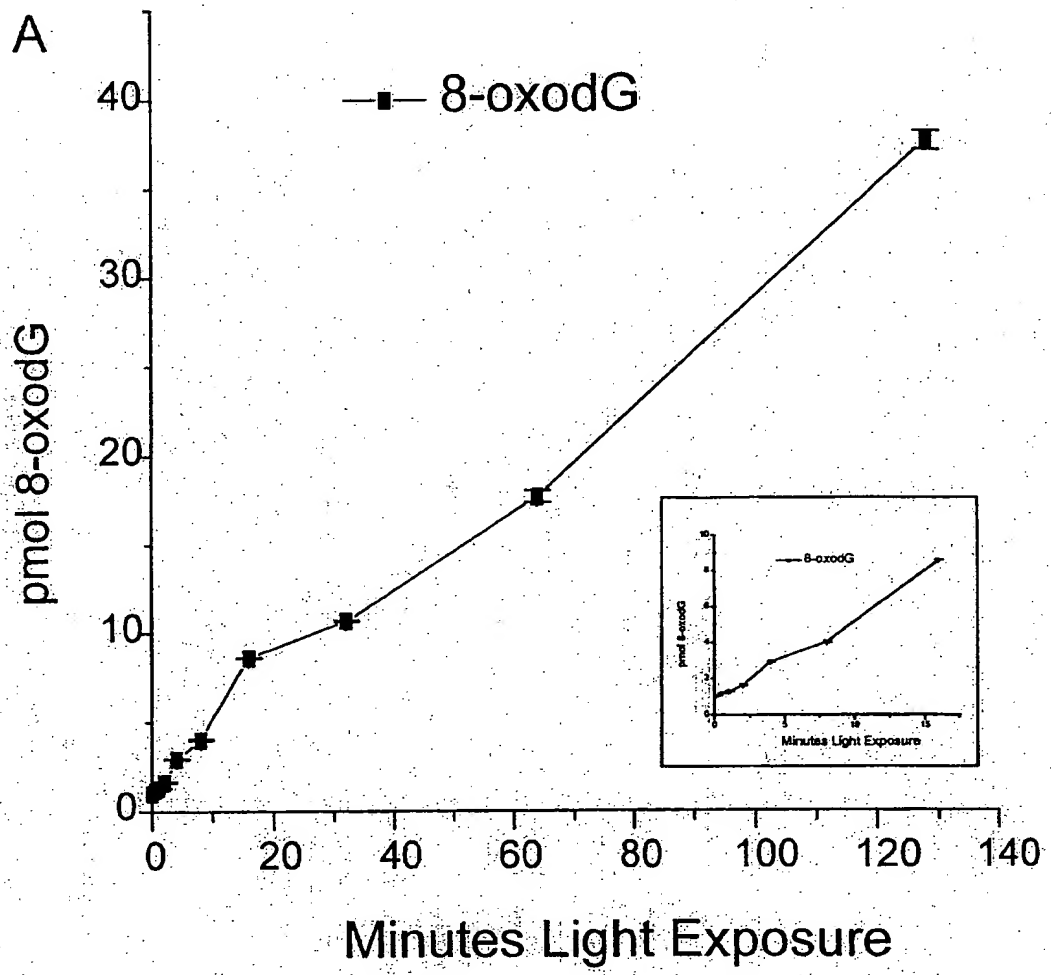

FIGURE 3 *HPLC-Coularray detection of 8-oxodG at physiological damage levels.* Photoactivated methylene blue (MB) was used to induce the lesion, 8-oxodG, in calf thymus DNA. DNA was exposed to MB for 0–120 min, then submitted blind for HPLC analysis. Channels 1 and 2 were set to detect 8-oxodG and channels 3 and 4 were set for 2-dG. The induction of damage remained linear up to 120 min (panel A) and was able to detect damage at physiological levels (Inset).

the 8-oxodG levels in the mtDNA were roughly four times higher than that in the nucleus. However, due to the age-associated increase in mtDNA damage, there was approximately 10 times more mitochondrial than nuclear DNA damage by 23 months.[25] The magnitude of the increase in mtDNA damage was in agreement with previous reports, however the absolute values were ten times lower.[15,25]

SUMMARY

The decrease in heart cytochrome oxidase activity is due to a decrease in mitochondrial protein synthesis and gene expression. Mitochondrial gene expression may in turn decrease as a result of mtDNA damage. mtODE activity increases with age in both rat heart and liver. In the liver, mitochondrial 8-oxodG content increases 2.5-fold

while there is no change in the level of damage in the nuclear DNA with age.

DISCUSSION

The measurement of oxidative damage in DNA has become an area of intense interest, and there is a portion of the scientific community that has dedicated itself to determining the most accurate method for the measurement of oxidative damage. We have used the HPLC combined with the electrochemical array to measure 8-oxodG as an indicator of the degree of oxidative damage. The relative changes in the levels of mtDNA damage are evident. There are numerous design advances that make the array the preferred method of analysis. The flow through property of the electrochemical cells allows complete oxidation of the sample and an accurate measurement. The coularray allows precise identification of peaks based on both retention time and the ratio of the current across multiple channels. This makes peak identification and quantitation more exact than single channel EC detectors. The advantage of HPLC–EC over GC–MS is the ease and relative mildness of the sample preparation for HPLC analysis reducing the likelihood of damage produced from handling and derivatization for GC–MS. However, it lacks the ability to measure a wide array of adducts.

The levels of 8-oxodG that we measured were approximately 10 times lower than those reported by other laboratories.[15,17] This is in large part due to the advances that Ames and his colleagues have made in the field of DNA isolation and nucleoside preparation.[20] This, in conjunction with the EC array technology, has served to "lower the bar" of DNA damage detection.

An exhaustive search of the literature produced relatively few original reports regarding the increased occurrence of mitochondrial DNA damage with age. The mitochondrial free radical theory of aging, for the most part, lacks extensive experimental support.[4] These results presented here can add to the inventory of mtDNA damage reports, but a comprehensive and long-ranged study in humans is needed to determine the true impact of free radical damage to mitochondria in aging.

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X. RELATED PROCEEDINGS APPENDIX

There are no related proceedings.